Vitamin E, LDL, and Endothelium

Brief Oral Vitamin Supplementation Prevents Oxidized LDL–Mediated Vascular Injury In Vitro

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In previously reported in vitro studies, we found that heme, a physiologically widespread hydrophobic iron compound, can rapidly generate oxidized low-density lipoprotein (LDL), which then becomes cytotoxic to cultured vascular endothelial cells; both LDL oxidation and endothelial cytotoxicity were inhibited by incubation with exogenous α-tocopherol (vitamin E) or ascorbic acid (vitamin C). Seeking relevance to in vivo conditions, we performed a study in which 10 human volunteers were given daily antioxidant supplements of 800 IU of DL-α-tocopherol acetate alone or in combination with 1000 mg of ascorbic acid for 2 weeks. LDL resistance to heme oxidation ex vivo, as measured by the lag time for conjugated-diene formation, increased by as much as threefold from a mean ± SD of 58 ± 11 to 194 ± 18 minutes (P < .001); LDL α-tocopherol increased from 11 ± 2 to 26 ± 6 molecules per LDL particle (P < .001); and most impressively, cytotoxicity to porcine aortic endothelial cells incubated with LDL conditioned with heme plus H2O2 or with copper was completely prevented (cytotoxicity before supplementation was 42 ± 12%, decreasing after supplementation to 3 ± 2%, P < .001). These measurements reverted to their presupplement levels within 2 weeks after participants stopped taking antioxidant supplements and were reproduced in 4 subjects taking 800 IU of DL-α-tocopherol acetate supplements alone but not in the same subjects taking 1000 mg ascorbic acid supplements alone. In conclusion, oral vitamin E supplementation increases LDL α-tocopherol content, increases LDL resistance to oxidation, and decreases the cytotoxicity of oxidized LDL to cultured vascular endothelial cells. (Arterioscler Thromb. 1993;13:1779-1789.)

**KEY WORDS** • vitamin E • LDL • oxidized LDL • vitamin C • atherosclerosis • heme • iron

High serum levels of low-density lipoprotein (LDL) cholesterol are associated with the development of coronary heart disease.1 LDL particles entering subendothelial “sanctuaries” of the artery wall can become trapped and exposed to oxidative stresses. LDL oxidation has been shown to foster recruitment of macrophages, and by binding to scavenger receptors on the surface of macrophages, oxidized LDL can ultimately generate foam cells.2-4 Oxidized LDL is also directly cytotoxic, particularly to vascular endothelial cells.5-7 Such damage would presumably exacerbate atheroma formation both by allowing LDL to freely enter the artery wall and by promoting platelet adherence and growth factor liberation.4,8

Contravening this deleterious process are several types of lipid-soluble antioxidants contained in LDL particles that protect LDL polyunsaturated fatty acids from oxidation.9 These include α- and γ-tocopherol, ubiquinol-10, and several members of the carotenoid family, primarily α- and β-carotene and lycopene. Also, diets high in monounsaturated fatty acids increase LDL resistance to oxidation in rabbits10 and humans.11-13 Ascorbic acid, a water-soluble antioxidant, can protect LDL from oxidative attack by aqueous free radicals14 and may also have a synergistic effect with α-tocopherol by reducing tocopherol radicals and thereby regenerating vitamin E.15

In epidemiological studies, dietary intake of vitamins C and E have been associated with a reduced risk of cardiovascular diseases in men and women.16-21 The risk of coronary heart disease was particularly low in men and women taking vitamin E supplements.20,21 suggesting that the inverse association between dietary antioxidants and cardiovascular disease could be related to the direct inhibition of LDL oxidation. LDL oxidation in vitro can be inhibited by adding α-tocopherol or ascorbic acid to isolated LDL.22 Dietary supplements of vitamin E inhibit LDL oxidation ex vivo22-24; the data on dietary vitamin C supplements are conflicting.22,26

Several other types of studies also have linked LDL oxidation to atherosclerosis. Oxidized LDL was found in atherosclerotic lesions of humans and LDL receptor-deficient rabbits.27,31 In addition, the titer of autoantibodies to malondialdehyde-LDL, an epitope found on oxidized LDL, was an independent predictor of the progression of carotid atherosclerosis.32 Finally, LDL susceptibility to oxidation, which can be measured in vitro by exposing isolated LDL to transition metals such as copper and iron, has also been associated with coronary atherosclerosis.33,34

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We recently demonstrated that LDL can be rapidly oxidized in vitro by heme, a hydrophobic, iron-containing compound. Heme is a physiologically ubiquitous compound that is particularly abundant in hemoglobin. From this we have hypothesized that heme might be a critical oxidant of LDL in vivo, particularly at sites downstream from areas of high wall shear stress, accompanied by red blood cell lysis (as at vascular bifurcations), or with hemorrhage into ruptured atherosclerotic plaques.

There are no previous data available regarding the effects of oral antioxidant supplements on LDL oxidation by heme or on the cytotoxicity of oxidatively modified LDL to cultured vascular endothelial cells. We report that very brief oral dosing with α-tocopherol acetate effectively doubles LDL α-tocopherol content and LDL resistance to oxidation by heme and dramatically decreases the cytotoxicity of oxidatively modified LDL to endothelial cells in culture. These protective effects, reported in preliminary form elsewhere, are manifested within 2 weeks of the start of α-tocopherol supplementation and are lost within 2 weeks of stopping supplementation.

Methods

Subjects

Ten healthy, nonsmoking participants (8 men and 2 women), aged 23 to 59 years, were recruited from among the faculty and staff working in the investigators’ laboratories, including the investigators themselves. The volunteers were instructed to maintain their normal diet and exercise patterns and to either not take any vitamin supplements or take 800 IU of DL-α-tocopherol acetate (vitamin E) per day after meals and/or 1000 mg of ascorbic acid (vitamin C) per day after meals. Participants took the vitamin supplements alone or in combination for 2 weeks. The supplementation periods were followed by washout periods lasting 2 weeks. Participants refrained from taking any vitamin supplements during the washout periods.

Materials

Hydrogen peroxide (H₂O₂) and potassium bromide (KBr) were obtained from Fisher Scientific, Pittsburgh, Pa. Bovine hemin type I, bovine serum albumin, EDTA, and Fluorinert (FC-40) were from Sigma Chemical Company, St Louis, Mo. Hanks’ balanced salt solution (HBSS), supplemented with sodium bicarbonate to provide physiological pH, was obtained from Gibco, Grand Island, NY. Ascorbic acid and dl-α-tocopherol acetate were manufactured by Walgreen Laboratories, Deerfield, Ill.

Preparation of Human LDL

Informed consent for drawing blood (60 mL) from the volunteers was obtained according to the guidelines of the Committee on Human Subjects at the University of Minnesota. Plasma LDL was prepared from EDTA (1 mg/mL)-anticoagulated venous blood after centrifugation of the blood at 2000g at 4°C for 20 minutes. Blood, plasma, and LDL samples were processed in subdued light on ice to inhibit photooxidation of LDL antioxidants. LDL was isolated from plasma by rate zonal density gradient ultracentrifugation. The density of plasma was raised to 1.3 g/mL by adding 5 g KBr to 10 mL of plasma. The KBr was dissolved in plasma by gentle mixing on a tipper for 10 to 15 minutes. Each KBr-plasma sample was layered underneath 23 mL of ice-cold 0.9% NaCl in an EasySeal polyallomer ultracentrifuge tube (Seton Scientific, Palo Alto, Calif). The sealed tubes were centrifuged at 50,000 rpm (302,000 × g) for 3 hours at 4°C in a 50.2 Ti fixed-angle rotor (Beckman Instruments, Palo Alto, Calif). After centrifugation, the density gradients were fractionated by piercing the bottom of the tube with a needle and pumping Fluorinert into the tube at a rate of 5 mL/min. The contents were collected from the top of the tube and diverted to a fraction collector (ISCO, Lincoln, Neb). The first 12 mL of the gradient, containing very low density lipoproteins (VLDL), was discarded. Subsequently, six fractions of 1.2 mL each were collected. The dark yellow LDL peak was generally found in the fourth fraction. LDL was pooled from the peak fraction plus one fraction after the peak and two fractions before the peak. This LDL gave a single β-migrating band after agarose electrophoresis, indicating that the LDL was free from contamination by other lipoproteins. LDL samples were dialyzed against 6 L of HBSS buffer, three times before use. The isolated LDL samples were stored on ice in the dark. The LDL cholesterol was measured enzymatically on a Synchro-CX5 autoanalyzer (Beckman Instruments, Brea, Calif). LDL protein content was determined by the Peterson-Lowry method with bovine serum albumin as a standard.

Measurement of LDL Resistance to Oxidation With Hemin and H₂O₂

LDL was oxidized with heme and H₂O₂ in 96-well Immulon 1 microtiter plates (Dynatech, Chantilly, Va) and in quartz cuvettes. The oxidation of LDL was monitored by measuring the decreasing absorbance of hemin at 405 nm in the microtiter assay and at 412 nm in the cuvette assay. The decrease in hemin absorbance parallels the increase in thiobarbituric acid–reactive substances (TBARS) and conjugated dienes. The final assay concentrations for the microtiter assay were 10 mg/dL LDL cholesterol, 2.5 μmol/L hemin, and 50 μmol/L H₂O₂ in N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)/NaCl (pH 7.4), in a final assay volume of 0.15 mL. LDL used for the microtiter assay was not dialyzed against HBSS buffer. This assay was always performed within 2 hours of LDL isolation and within 8 hours of blood collection. The final concentrations for the cuvette assay were 400 μg/mL LDL protein (postdialysis), 10 μmol/L hemin, and 50 μmol/L H₂O₂ in HBSS buffer, pH 7.4, in a final assay volume of 2 to 3 mL. The assays were started by the addition of H₂O₂. Each LDL sample was assayed in quadruplicate for the microtiter assay and once for the cuvette assay. After the addition of H₂O₂, the plate was read at 43-second intervals for 4 hours in a Vmax kinetic microtiter plate reader (Molecular Devices, Menlo Park, Calif), and the cuvette was read at 10-minute intervals for 4 hours in a Beckman DU-70 spectrophotometer (Beckman Instruments). The resistance of LDL to oxidation was measured by the lag time in minutes between the start of the assay (H₂O₂ addition) and the start of the propagation phase as determined by hemin absorbance. The start of the propagation phase is marked by a rapid decrease in hemin absorbance. In some LDL samples (eg, Fig 4), the resistance of LDL to oxidation was measured as the time required for the reaction to reach maximum velocity (ie, time to Vmax). Vmax, as measured by the change in hemin...
absorbance, was reached near the midpoint of the propagation phase. The time to \(V_{\text{max}}\) was computed by computer software linked to the plate reader (Molecular Devices, Menlo Park, Calif). The correlation \(r\) between lag time and time to \(V_{\text{max}}\) in 56 samples was 0.992; however, time to \(V_{\text{max}}\) was approximately 10 minutes longer than lag time. The analytic and analytic plus biological coefficients of variation for measuring lag time by the microtiter method were 5.9% and 9.6%, respectively.

**Preparation of Hemin Solutions**

A fresh stock solution of hemin (1 mmol/L) was prepared daily by dissolving hemin in 0.02 mol/L NaOH. A working solution of hemin was prepared by diluting the stock with either ice-cold HEPES/NaCl (10/150 mmol/L) buffer, pH 7.4 (microtiter assay), or HBSS buffer, pH 7.4 (cuvette assay).

**Preparation of \(H_2O_2\) Solutions**

A dilute stock solution of 1.5 mmol/L \(H_2O_2\) was prepared by diluting 0.167 mL of 30% \(H_2O_2\) to a final volume of 1 L with distilled water. This dilute stock was stored in the dark in a refrigerator. A working solution of \(H_2O_2\) was prepared fresh daily by diluting the stock solution of \(H_2O_2\) with ice-cold HEPES/NaCl (10/150 mmol/L) buffer, pH 7.4 (microtiter assay), or HBSS buffer, pH 7.4 (cuvette assay).

**Measurement of LDL Resistance to Oxidation With Copper Sulfate (\(CuSO_4\))**

In some experiments LDL was oxidized with \(CuSO_4\) for comparison to LDL oxidized with hemin and \(H_2O_2\). The final concentrations for the assay were 400 \(\mu\)g/mL LDL protein and 10 \(\mu\)mol/L \(CuSO_4\) in HBSS buffer, pH 7.4, in a final assay volume of 1 mL. A 1 mmol/L \(CuSO_4\) stock solution was prepared in distilled water just before the experiments and was used immediately to catalyze the LDL oxidation. The assays were started by the addition of \(CuSO_4\). The production of conjugated dienes was monitored at 234 nm at 10-minute intervals for 4 hours in a Beckman DU-70 spectrophotometer. The resistance of LDL to oxidation was measured by the lag time in minutes between the start of the assay and the start of the propagation phase as determined by conjugated-diene production.

**Oxidation of LDL for Endothelial Cell Experiments**

LDL was oxidized by hemin and \(H_2O_2\) in HBSS for the porcine aortic endothelial cell (PAEC) killing experiments. The final concentrations for LDL oxidation were 400 \(\mu\)g/mL LDL protein, 10 \(\mu\)mol/L hemin, and 50 \(\mu\)mol/L \(H_2O_2\) in a final assay volume of 1.5 mL. The assays were started by the addition of \(H_2O_2\). In a few experiments, LDL was oxidized with \(H_2O_2\) (final concentration) in HBSS. The LDL was incubated with either hemin/\(H_2O_2\) or \(CuSO_4\) for 45 minutes at 37°C before the PAEC killing experiments.

**Isolation and Culture of PAECs**

PAECs were isolated enzymatically from porcine aortas by use of type I collagenase as previously described.7 The cultures were used from passages 3 to 7 and studied within 48 hours of reaching confluence.

**PAEC Cytotoxicity Assays**

The cytotoxicity of normal and oxidatively modified LDL to PAECs was determined by \(^{51}Cr\) release as previously described.7 The control cells were incubated for 4 hours with 1 mL HBSS, and the others were incubated for 4 hours with normal or oxidatively modified LDL (200 \(\mu\)g/mL protein) in HBSS. Spontaneous \(^{51}Cr\) release was <20% in all experiments.

**Measurement of TBARS**

After oxidative modification of LDL (400 \(\mu\)g/mL LDL protein concentration), 300 \(\mu\)L of the incubation mixture was added to 600 \(\mu\)L thiobarbituric acid reagent (0.375 g of 2-thiobarbituric acid, 2.08 mL 12N HCl, 15 mL 100% trichloroacetic acid, and distilled water to a final volume of 100 mL). Butylated hydroxytoluene (10 mg/mL) dissolved in dimethyl sulfoxide was added to a final concentration of 0.1 mg/mL to inhibit spontaneous formation of TBARS during the subsequent heating step. After heating at 100°C for 15 minutes, the samples were cooled to room temperature and centrifuged at 10,000g for 10 minutes. The clear supernatants were analyzed spectrophotometrically at 532 nm with an extinction coefficient of 1.56X10^5 mol/L^1·cm^−1, and the results are presented as nanomoles TBARS per milligram LDL protein.

**Measurement of \(\alpha\)-Tocopherol**

The \(\alpha\)-tocopherol content of LDL was determined by the method of Craft et al39 with minor modifications. Protein was precipitated with ethanol and the sample extracted with hexane. The residue was dried and extracted with methanol and the organic extracts were combined for further analysis. An internal standard of tocopherol acetate was added to each sample before extraction. The internal standard was used for the determination of tocopherol recovery and quantitation. The extract was analyzed on a System Gold high-performance liquid chromatograph (Beckman Instruments, Irvine, Calif) equipped with a Supelcosil C18 column (Supelco, Bellefonte, Pa). External tocopherol standards and a pooled plasma sample were analyzed with each set of samples as part of daily quality control procedures to ensure precise quantitation.

**Apo protein B (Apo B)**

Apo B was measured in purified LDL by a kinetic immunonephelometric assay to calculate the number of molecules of \(\alpha\)-tocopherol per LDL particle. The assay was calibrated against human sera obtained from the Centers for Disease Control and Prevention, Atlanta, Ga. An apo B molecular weight of 550 000 g/mol and a stoichiometry of 1 apo B molecule per particle were used to calculate the concentration of LDL particles. The number of \(\alpha\)-tocopherol molecules per LDL particle was calculated as the molar ratio of \(\alpha\)-tocopherol to apo B.

**Statistics**

Significance of changes was determined by Student’s paired \(t\) test. Mean levels were computed for each variable at each phase of the study. Changes were computed for each participant after 2 weeks of supplementation compared with presupplementation and at 2 weeks after cessation of supplementation (wash-out) compared with after 2 weeks of supplementation.
Results

Typical LDL Oxidation Curve

Detailed studies of heme absorbance, conjugated-diene absorbance, and TBARS production were made in 3 subjects. Heme and H₂O₂ together catalyze the oxidation of LDL in vitro (Fig 1). The reaction kinetics of heme/H₂O₂ are analogous to copper-mediated oxidation of LDL and consist of three phases. The first phase of the reaction is a lag phase that corresponds to consumption of LDL antioxidants. The second phase is a propagation phase that corresponds to the oxidation
of polyunsaturated fatty acids; this phase produces conjugated dienes and TBARS in parallel with decrease in heme absorbance at 412 nm. The third phase is the decomposition, or termination phase, which is associated with stabilization of heme absorbance and a decrease in conjugated dienes and TBARS. LDL from subject 1, who was not taking any oral antioxidant supplements, had a short lag time (40 minutes). LDL from subject 2, who was taking 400 IU of DL-a-tocopherol acetate every third day for several months, had an intermediate lag time (100 minutes). LDL from subject 3, who was taking 400 IU of DL-a-tocopherol acetate daily for several months, had the longest lag time (200 minutes). During heme-mediated LDL oxidation, the easily assayable loss of the heme Soret-band absorbance at 412 nm closely parallels the formation of conjugated dienes and TBARS (Fig 1). Thus, we now routinely measure heme absorbance change as a surrogate assay for LDL oxidation in 96-well microtiter plates.

Comparison of Lag Times for Copper- and Heme-Mediated LDL Oxidation

LDL oxidation catalyzed by copper or heme was compared in 30 samples (Fig 2). The lag times for copper-mediated and heme-mediated LDL oxidation were highly correlated (r = .929). The linear regression slope and intercept were 0.96 and 7.0 minutes, respectively. LDL (400 μg/mL protein) was oxidized by either 10 μmol/L CuSO4 or 10 μmol/L hemin plus 50 μmol/L H2O2. Lag times were calculated for both methods by using conjugated-diene formation measured at 234 nm.

Supplementation Experiment 1

To further test the effects of a-tocopherol, 9 human volunteers were asked to abstain from vitamin supplements for at least 4 weeks before the study. In addition, 1 of the 9 volunteers repeated the experiment 1 year later. After the presupplement period, the participants took 800 IU of DL-a-tocopherol acetate per day alone (n = 4) or in combination with 1000 mg of ascorbic acid supplements per day (n = 6) for 2 weeks. After 2 weeks of antioxidant supplementation, the volunteers began a 2-week washout period without supplements. EDTA-anticoagulated blood samples were collected immediately before starting the supplements (presupplement), after 2 weeks on supplements (+a-tocopherol), and after 2 weeks off supplements (washout). We isolated LDL from each subject’s plasma and measured the number of a-tocopherol molecules per LDL particle (Fig 3A), LDL resistance to oxidation (Fig 3B), and the cytotoxicity of heme/H2O2-conditioned LDL to PAECs (Fig 3C). LDL a-tocopherol content and LDL resistance to oxidation increased in all subjects from a mean of 11 ± 2 molecules per LDL particle and 58 ± 11 minutes, respectively, at baseline to a mean of 26 ± 6 molecules per LDL particle (P < .001) and 104 ± 18 minutes (P < .001), respectively, after 2 weeks of supplementation. Two weeks after subjects stopped taking the supplements, LDL a-tocopherol levels and LDL resistance to oxidation almost returned to their baseline levels (10 ± 3 molecules per LDL particle, P > .001, and 67 ± 10 minutes, P > .001). In addition, the rate of the propagation phase during LDL oxidation was 36% slower in subjects taking the supplements compared with baseline or washout values (not shown). The cytotoxicity of heme/H2O2-conditioned LDL to PAECs was high at baseline (42 ± 12%, mean ± SD). The cytotoxicity decreased in all subjects’ LDL after 2 weeks of a-tocopherol with or without ascorbic acid supplementation (3 ± 2%, P < .001). Two weeks after the subjects stopped taking the supplements, the cytotoxicity had almost returned to baseline levels (40 ± 5%, P < .001). Comparable results (not shown) were obtained with CuSO4 to precondition the LDL before PAEC incubations; in 3 individuals, PAEC cytotoxicity was 52 ± 4% before a-tocopherol supplementation and decreased to 9 ± 8% after 2 weeks of vitamin E ingestion.

Supplementation Experiment 2

Next, 5 of the volunteers were given supplements of either a-tocopherol alone or ascorbic acid alone to determine the independent effects of a-tocopherol and ascorbic acid supplements on LDL a-tocopherol content, LDL resistance to oxidation, and heme/H2O2-conditioned LDL cytotoxicity to endothelial cells. After abstaining from vitamin supplements for 2 weeks, 5 volunteers took either 1000 mg of ascorbic acid per day for 2 weeks or 800 IU of a-tocopherol acetate per day for 2 weeks (4 of 5 volunteers). Three of the subjects started taking ascorbic acid supplements and the other 2 started taking a-tocopherol supplements. After taking the vitamin supplements for 2 weeks, the participants stopped taking the supplements for 2 weeks (washout). After the 2-week washout, the participants crossed over to the other antioxidant supplement for an additional 2 weeks. The second 2-week supplementation period was followed by another 2-week washout period. EDTA-blood samples were collected before and after each 2-week period. In a few participants (as indicated in Fig 4), additional blood samples were collected after only 1 week.
Fig 3. Facing page. Bar graphs showing effects of brief oral supplementation with α-tocopherol: A, increases in low-density lipoprotein (LDL) α-tocopherol content, B, increases in LDL resistance to heme/H2O2-mediated oxidation ex vivo, and C, protection of cultured porcine aortic endothelial cells from the cytotoxic effects of LDL conditioned with heme/H2O2. Protective effects of α-tocopherol (800 IU per day) were manifested 2 weeks after beginning supplementation and were rapidly lost within 2 weeks of stopping α-tocopherol supplementation. Six subjects (first six bars from left) were also taking ascorbic acid supplements with the α-tocopherol supplements. One of the 6 volunteers repeated the experiment 1 year apart with (bar 6 from left) and without (bar 7 from left) ascorbic acid supplements. Cytotoxicity results represent mean±SEM of two cell-culture incubations. LDL resistance to oxidation was measured in quadruplicate on microtiter plates.

Discussion

We have previously demonstrated that heme, a ubiquitous, hydrophobic, iron-containing compound, readily intercalates into LDL particles and can rapidly oxidize LDL in vitro.7 Heme-catalyzed oxidation of LDL is accelerated by activated inflammatory cells, small amounts of H2O2, or preformed lipid hydroperoxides within the LDL. We have also observed that heme-conditioned LDL is markedly cytotoxic to cultured endothelial cells.7 The present studies demonstrate that brief oral supplementation with α-tocopherol inhibits heme/H2O2-mediated and copper-mediated LDL oxidation ex vivo and protects cultured endothelial cells from the cytotoxic effects of such conditioned LDL. The protective effects of α-tocopherol were manifested 1 to 2 weeks after beginning supplementation and were rapidly lost within 2 weeks of stopping α-tocopherol supplementation. In ancillary studies (not shown) we also observed a similarly rapid washout of α-tocopherol, even in subjects who were taking 400 IU of α-tocopherol daily for several months. We7 and others14-42 have previously shown that exogenous ascorbic acid is a potent inhibitor of LDL oxidation by heme/H2O2, copper, or aqueous free radicals in vitro. However, in the present studies oral supplementation with ascorbic acid was without beneficial effect on LDL resistance to oxidation ex vivo. It is known that in vitro, ascorbic acid and α-tocopherol act synergistically, in that ascorbic acid can reduce α-tocopherol after its oxidation.15 Ascorbic acid is also very effective in inhibiting the formation of lipid hydroperoxides in LDL exposed to aqueous free radicals.14 Thus, we thought it likely that oral supplementation with ascorbic acid might increase LDL resistance to oxidation ex vivo by sparing LDL α-tocopherol or by decreasing preformed lipid hydroperoxides in LDL. However, in our study oral supplements of ascorbic acid, when provided alone or in combination with supplemented α-tocopherol, did not increase LDL resistance to oxidation ex vivo or LDL α-tocopherol content; moreover, ascorbic acid did not decrease the cytotoxicity of oxidatively modified LDL to PAECs.

Our findings on ascorbic acid should be interpreted with caution because they were generated in LDL that was oxidized ex vivo in the absence of its normal ascorbic acid environment. That is, ascorbic acid is a water-soluble antioxidant that might be removed during LDL isolation from plasma. Nevertheless, it seems likely that lipid-rich atherosclerotic lesions may also represent microenvironments where concentrations of hydrophilic ascorbic acid might be insufficient to protect LDL from oxidation. Under these conditions, LDL would need to rely on its own endogenous lipophilic antioxidants, such as α-tocopherol, for protection.

Another caveat concerning ascorbic acid derives from the fact that all of the participants in this study were nonsmokers. It is conceivable that ascorbic acid supplements might be more effective in increasing LDL resistance to oxidation ex vivo or in smokers, since their normal ascorbic acid levels are abnormally low blood ascorbic acid levels.43 Moreover, cigarette smoke generates copious amounts of free radicals, a notion that perhaps underlies the results demonstrating that acute smoking potentiates plasma LDL oxidation,25-46 which is inhibited by either α-tocopherol or ascorbic acid supplementation.22

Recently others have performed related studies that agree with ours. Reaven and colleagues26 reported that long-term supplementation with large doses (1600 mg/d) of vitamin E alone but not β-carotene or vitamin C conferred increased protection against LDL oxidation ex vivo. Addition of vitamin E increased LDL vitamin E levels approximately 2.5-fold and increased LDL lag time during copper-mediated oxidation by 1.5-fold. The rate of the propagation phase was also significantly slower in the vitamin E–treated subjects. Dieber-Roth et al23 supplemented eight volunteers with 150, 225, 800, or 1200 IU of α-tocopherol for 21 days. The LDL content of α-tocopherol increased by 1.7- to 3.1-fold, and LDL lag time increased by 1.2- to 2.3-fold, similar to the findings in our study in which LDL α-tocopherol increased by 1.4- to 3.5-fold (mean, 2.3-
Fig 4. Line plots showing effects of α-tocopherol supplements taken alone, but not ascorbic acid supplements taken alone: (1) increases in low-density lipoprotein (LDL) α-tocopherol content; (2) increases in LDL resistance to heme/H$_2$O$_2$-mediated oxidation ex vivo; and (3) protection of porcine aortic cultured endothelial cells from the cytotoxic effects of LDL conditioned with heme/H$_2$O$_2$. Subjects took either 800 IU of dl-α-tocopherol acetate per day for 2 weeks (A, C, and E) or 1000 mg of ascorbic acid per day for 2 weeks (B, D, and F). The 2-week supplementation period was followed by a 2-week washout period with no antioxidant supplementation. Blood was collected and analyzed weekly in some subjects and biweekly in others as indicated by the symbols. Four of 5 subjects participated in both supplement experiments; half took α-tocopherol first and half took ascorbic acid first. Subjects began taking the second antioxidant immediately after the first 2-week washout period.
fold) and LDL lag time increased by 1.2- to 2.8-fold (mean, 1.8-fold). In addition, Princen et al.23 gave oral supplements of α-tocopherol and β-carotene to nonsmokers and smokers and assayed LDL resistance to copper-mediated oxidation. No changes in LDL resistance to oxidation between the β-carotene group and the placebo group were detected, but nonsmokers supplemented with 1000 IU of α-tocopherol acetate per day for 7 days were significantly modulated: LDL α-tocopherol increased 2.4-fold and LDL lag time increased 1.4-fold. Moreover, a strong correlation between the increase in LDL resistance time and the increase in LDL α-tocopherol content was found (r=.89), but no correlation was evident cross-sectionally in nonsupplemented participants. Likewise, our studies have found significant correlations between the increase in LDL lag time and the increase in LDL α-tocopherol during supplementation (r=.52, P<.05) and the decrease in LDL lag time and the decrease in LDL α-tocopherol during washout (r=.66, P<.05). In agreement with several other reports,23,24,47,48 we found no correlation between LDL lag time and LDL α-tocopherol content cross-sectionally in nonsupplemented participants.

Although several reports have demonstrated beneficial effects of oral antioxidant supplementation on LDL resistance to copper-induced oxidation,22-24,47 the present findings are novel in three respects. First, heme induction of LDL oxidation was used, which may be more relevant to atherogenesis in vivo. Second, the ability of oral vitamin E supplementation to protect cultured endothelial cells from the cytotoxic action of oxidatively modified LDL was assayed; to our knowledge this is the first time this system has been used to validate the beneficial effects of vitamin supplementation. Third, the time course of α-tocopherol-mediated LDL protection and washout of its effects, particularly on endothelial cytotoxicity, was evaluated.

It is not known how LDL oxidation is initiated in vivo. Metabolic products of endothelial cells, smooth muscle cells, and monocytes/macrophages can oxidize LDL in the presence of iron or copper.23,24 Others have shown that iron and copper accumulate in atherosclerotic lesions in a catalytically active form and that these binding proteins under normal circumstances probably bind most of the free heme before it can intercalate into LDL or endothelial cell membranes. However, our data suggest that this extracellular protection can be overwhelmed: cultured endothelium can successfully compete for free heme and hemoglobin with these heme-liganding proteins.53

Oxidative modification of LDL, if in fact it does take place in the artery wall, must occur under a very complex set of conditions affected by plasma components that are transported or infiltrated into the subendothelial space. So far as we are aware, the composition and concentration of lipoproteins, proteins, and antioxidants in the subendothelial space are unknown and probably highly variable, depending on plasma concentrations and endothelial function and integrity. If LDL becomes oxidized within the artery wall, it could promote atherogenesis by several possible mechanisms.4 These mechanisms include foam cell formation, cytotoxicity to endothelial cells, stimulation of growth factor expression, elicitation of autoantibody formation, alteration of coagulation pathways, and inhibition of vasodilation pathways. Vitamin E could potentially attenuate all of these pathways by inhibiting LDL oxidation either directly or indirectly by inhibiting the cellular production of free radicals, which can oxidize LDL.

Several epidemiological studies have reported a protective effect of vitamin E on coronary heart disease. In cross-cultural epidemiological studies of European men, lipid-standardized vitamin E levels (α-tocopherol to cholesterol ratio) were the most important risk factor (inverse) for ischemic heart disease.18 In a case-control study of men in Edinburgh, low plasma concentrations of vitamins A, C, and E and carotene were associated with increased risk for angina pectoris.19 In a cohort of 39,910 men being studied at Harvard, men in the highest compared with the lowest quintiles of vitamin E and carotene intake had a lower relative risk of coronary heart disease. When the contributions of vitamin E from foods and supplements were examined, only vitamin E supplement users were at lower risk.20 Similar observations were reported for a cohort of US female nurses.21 The present study, particularly the endothelial cytotoxicity results, may provide pathophysiological insight into these epidemiological observations. It also provides guidelines for supplementation and washout times (approximately 14 days) for further vitamin E prevention trials that, given the currently available evidence and the lack of significant side effects associated with megavitamin E supplementation,34 seem eminently worthwhile.

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Vitamin E, LDL, and endothelium. Brief oral vitamin supplementation prevents oxidized LDL-mediated vascular injury in vitro.

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