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Oral α-tocopherol Supplementation Inhibits Lipid Oxidation in Established Human Atherosclerotic Lesions

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Background: Much experimental evidence suggests that lipid oxidation is important in atherogenesis and in epidemiological studies dietary antioxidants appear protective against cardiovascular events. However, most large clinical trials failed to demonstrate benefit of oral antioxidant vitamin supplementation in high-risk subjects. This paradox questions whether ingestion of antioxidant vitamins significantly affects lipid oxidation within established atherosclerotic lesions.

Methods and results: This placebo-controlled, double blind study of 104 carotid endarterectomy patients determined the effects of short-term α-tocopherol supplementation (500 IU/day) on lipid oxidation in plasma and advanced atherosclerotic lesions. In the 53 patients who received α-tocopherol there was a significant increase in plasma α-tocopherol concentrations (from 32.66 ± 13.11 at baseline to 38.31 ± 13.87 (mean ± SD) μmol/l, $p < 0.01$), a 40% increase (compared with placebo patients) in circulating LDL-associated α-tocopherol ($p < 0.0001$), and their LDL was less susceptible to *ex vivo* oxidation than that of the placebo group (lag phase 115.3 ± 28.2 and 104.4 ± 15.7 min respectively, $p < 0.02$). Although the mean cholesterol-standardised α-tocopherol concentration within lesions did not increase, α-tocopherol concentrations in lesions correlated significantly with those in plasma, suggesting that plasma α-tocopherol levels can influence lesion levels. There was a significant inverse correlation in lesions between cholesterol-standardised levels of α-tocopherol and 7β-hydroxycholesterol, a free radical oxidation product of cholesterol.

Conclusions: These results suggest that within plasma and lesions α-tocopherol can act as an antioxidant. They may also explain why studies using <500 IU α-tocopherol/day failed to demonstrate benefit of antioxidant therapy. Better understanding of the pharmacodynamics of oral antioxidants is required to guide future clinical trials.

Keywords: Carotid (human); Atherosclerosis; Lipids; Oxysterol; α-Tocopherol

INTRODUCTION

Over recent years numerous *in vitro* and *in vivo* experimental studies have suggested a role for lipid peroxidation in the pathogenesis and progression of atherosclerosis.[1] The lipid oxidation theory of atherogenesis[1–3] is based on the observation that oxidation of LDL enhances its affinity for the macrophage scavenger receptor leading to increased LDL uptake and macrophage activation. Activated macrophages, through erosion of the fibrous cap and cytotoxic actions on vascular smooth muscle cells, are thought to be the main mediators of plaque rupture and hence clinical events.[4–6] The logical corollary is that increased plaque antioxidant activity will protect against plaque rupture.

Supporting this hypothesis, epidemiological studies, both between and within populations, have demonstrated an inverse correlation between dietary antioxidants and/or circulating levels of antioxidant vitamins and the incidence of coronary events.[7] Thus there was a realistic expectation that dietary

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supplementation with antioxidant vitamins would reduce the risk of future coronary events in high-risk populations, which prompted various clinical trials. Although the Cambridge Heart Antioxidant Study (CHAOS) suggested that vitamin E therapy reduced the rate of non-fatal myocardial infarction in patients with established coronary artery disease,[8] several other large studies including GISSI, HOPE and the Heart Protection Study failed to demonstrate any beneficial effect of antioxidant vitamins against major vascular events.[9–11] This paradox is unexplained, but oral antioxidant vitamins might not necessarily be beneficial against lipid oxidation within established, advanced atherosclerotic lesions.

A considerable gap seems to remain between the largely supportive epidemiological and experimental findings and whatever are the real events occurring within the human arterial wall. In an attempt to bridge this gap, we have investigated some of the relevant chemical findings in plasma samples and atherosclerotic lesions, in a randomised placebo-controlled study of short-term oral α-tocopherol (vitamin E) supplementation, in a total of 104 patients undergoing endarterectomy for symptomatic carotid artery disease.

METHODS

Patients and Samples

Unselected consecutive patients attending the Addenbrooke's Hospital Transient Ischaemic Attack (TIA) Clinic with recent symptoms of a TIA and a >70% carotid artery stenosis on the appropriate side, angiographically confirmed, were invited to participate in the study during the outpatient visit at which they were placed on the waiting list for carotid endarterectomy. Addenbrooke's Hospital Ethical Committee approved the study. Patients who gave written, informed consent to participate were randomised to receive either 500 IU α-tocopherol (TOCO-500™), or matching placebo, orally, once daily with a meal in a double blind fashion. The active ingredient in the capsules was all-rac-α-tocopheryl acetate (500 mg, i.e. 500 IU, per capsule). For placebo, liquid paraffin (442 mg per capsule) was used instead of active ingredient. The capsule material itself was composed of gelatin (46% w/w), glycerol (18% w/w), purified water (36%) and preservatives: ethyl parahydroxybenzoate (0.2% w/w), propyl parahydroxybenzoate (0.1% w/w) and chlorophyllin Na–Cu (0.01% w/w; this is also a colorant). The boxes of capsules were numbered by the suppliers (Laboratories Negma/Pharma 2000), enabling unblinding after all of the patients' specimens had been analysed. Compliance was assessed by capsule counting.

A plasma sample (non-fasting) was taken on recruitment day (plasma A) and frozen at −70°C within 1 h. A second sample (fasting) was taken in theatre on operation day (plasma B) and frozen at −70°C immediately.

Carotid endarterectomy specimens were refrigerated (4°C) then processed within a few hours of operation and stored at −80°C under argon. In the first 48 patients, a large portion of the atherosclerotic lesion was taken, including fibrous cap and underlying gruel. In all subsequent patients, the whole lesion was homogenised (using an IKA Ultra Turrax T8).

Analytical Methods

The tissue sample was thawed and weighed. Antioxidant (butylated hydroxytoluene, 50 μg per specimen) was added, and homogenised with 5 ml saline (0.9% w/v). The sample was then sub-divided for the analyses below.

Lipid and Oxysterol Profiling by GC

Internal standards (heptadecanoic acid, coprostane and 5α-cholestane; typically 96, 13.5 and 78 μg, respectively per sample) were added to the sub-sample, its volume was adjusted to 4 ml using saline (0.9%) and it was extracted by the Bligh and Dyer method as described previously.[12] For the non-homogenised samples, sonication (on ice) was included at this extraction stage.[12] The rest of the processing, comprising sodium borohydride reduction, saponification and derivatisation, was as described previously.[12,13] Processed samples were stored in hexane under argon at −20°C.

Analysis was on a Carlo Erba GC8000 with a cold on-column injector, hydrogen carrier gas (80 kPa), a DB-1 capillary column (30 m × 0.32 mm, film 0.1 μm) (J&W Scientific, Folsom, CA) and a flame ionisation detector, with a Shimadzu CR3A integrator. The temperature programme was as described previously.[14] Components were quantified by peak areas relative to the internal standards. Levels of individual components were expressed relative to wet weight of lesion material or, for plasma, relative to volume, and also relative to cholesterol in both cases.

Tocopherol Measurement by HPLC

Internal standard (α-tocopheryl acetate, 30 μg) was added to the sub-sample, the volume was adjusted to 3 ml with HPLC-grade water and mixed. SDS (1 ml of a 50 mM solution in HPLC-grade water, omitted in the case of plasma), acetonitrile (2 ml), ethanol (3 ml) and hexane (4 ml) were then added, vortexed well, and centrifuged. The hexane layer was collected,

evaporated under nitrogen and redissolved in methanol. HPLC analysis was on a Hewlett Packard 1050 with a Spherisorb ODS-2 column (25 cm × 4.6 mm) eluted with methanol (2 ml/min), with a UV/vis detector (292 nm) and a Hewlett Packard ChemStation data system. α-Tocopherol was quantified by peak areas relative to the α-tocopheryl acetate internal standard, with reference to standard curves of α-tocopherol plus α-tocopheryl acetate processed in parallel. Alpha-tocopherol levels were expressed as nmol/g wet weight of lesion or μmol/l plasma, and also relative to cholesterol, i.e. "cholesterol-standardised" as mmol/mol cholesterol, in both cases.

Measurement of LDL-associated α-tocopherol and of LDL Oxidisability Ex Vivo (Diene Conjugation Assay)

LDL was isolated from plasma by ultracentrifugation.[15] The LDL isolates were standardised using a cholesterol assay[16] (kit from Sigma; cat. no. 352-20). The α-tocopherol content of the isolated LDL was measured by HPLC as above, and expressed as mmol α-tocopherol/mol LDL cholesterol. The oxidisability of the LDL was measured by the diene conjugation assay,[16,17] as follows. LDL samples were diluted to 75.2 mg LDL cholesterol/ml in phosphate-buffered saline (PBS) and were oxidised with 1.6 μM copper (II) sulphate in quartz cuvettes maintained at 30°C in a spectrophotometer (Helios α, Unicam) monitoring absorbance at 234 nm every 5 min, for 240 min. Three A–B pairs (see above) were analysed concurrently, with a control (LDL without Cu^{2+}). Absorbance (*y*-axis) vs. time (*x*-axis) followed a sigmoid-like curve. Parameters calculated (using Excel software) were "slope" (absorbance units/minute) which was the gradient of the steepest part of the propagation phase, "lag phase" which was the intercept of the "slope" straight line with the *x*-axis (minutes), and "peak" (absorbance units) which was the highest point of the curve.

Agarose Gel Electrophoresis of Plasma

Aliquots of plasma (4 μl) were run on agarose gel electrophoresis (Beckman Paragon LipoGel) and stained with Sudan Black B (Beckman Lipostain), according to the manufacturer's instructions. Densitometry of the gels to evaluate the relative proportions of the bands was performed using Quantiscan software (Biosoft, Cambridge, UK). Plasma A–B pairs were run simultaneously. A reference mixture of LDL and HDL was run alongside at a range of dilutions. Relative proportions of bands were expressed as the equivalent cholesterol ratios based on the reference mixture.

Statistical Analysis

The study was unblinded once all biochemical measurements were complete. Data were analysed using StatView and Excel software, by paired Student's *t*-test (for comparisons within-patient, e.g. comparison of plasma A with plasma B) by unpaired Student's *t*-test for comparison of the α-tocopherol treated (TOCO; $n = 53$) and placebo (PLAC; $n = 51$) patient groups, Kendall's rank correlation, and, in selected cases, linear regression and correlation coefficient r^2. The level of significance was $p < 0.05$. Correlations are positive (i.e. two parameters increase concomitantly) unless stated to be negative, also termed inverse (i.e. as one parameter increases, the other parameter decreases). Positive and negative values of tau indicate positive and inverse trends, respectively. All " ± " data are mean ± standard deviation (SD).

To assess the results for the possible influence of statins, prescribed in some cases during the study, the patients were further sub-grouped retrospectively as follows: nonstatin plus α-tocopherol (NSTOC; $n = 37$), statin plus α-tocopherol (STTOC; $n = 16$), nonstatin plus placebo (NSPLAC; $n = 36$) and statin plus placebo (STPLAC; $n = 15$), collectively termed "the 4 patient sub-groups" hereafter. The ANOVA *post hoc* test Fisher's PLSD (StatView software) was used for multiple comparisons between the 4 patient sub-groups, with $p < 0.05$ as the level of significance. Correlation tests were as above.

RESULTS

One hundred and four patients (73 males; 31 females) aged 28–83 years (mean 68.6 ± 8.1 y) were randomised in the study. Fifty-one patients received placebo (PLAC group) and 53 received α-tocopherol (TOCO group). The median time from recruitment to operation was 78 days (range 8–217 d, mean 85 ± 36 d). Eighty patients received more than 60 days' treatment pre-operatively. Thirty-one patients were prescribed statin therapy during the study, 16 of whom received α-tocopherol and 15 placebo. All patients completed the protocol and capsule counts indicated good compliance.

For Plasma A, which was taken at recruitment, there were no significant differences between the TOCO and PLAC groups for α-tocopherol levels in plasma and LDL (Tables I and II), plasma cholesterol levels (Table I), plasma fatty acid levels, LDL oxidisability (Table II), or for relative proportions of LDL and HDL bands (LDL/HDL ratio) on electrophoresis. These findings were also true for the 4 patient sub-groups.

TABLE I Composition of plasma and lesions

Patient treatment group	Sample	Total cholesterol mmol/l plasma or μmol/g wet weight lesion	Total α-tocopherol	
			μmol/l plasma or nmol/g wet weight lesion	mmol/mol cholesterol
TOCO	Plasma A	5.16 (±1.13)	32.66 (±13.11)	6.34 (±1.98)
PLAC	Plasma A	5.37 (±1.30)	31.84 (±9.60)	6.02 (±1.33)
TOCO	Plasma B	$4.30^{\ddagger}$ (±1.09)	$38.31^{*,\dagger}$ (±13.87)	$9.15^{*,\ddagger}$ (±3.56)
PLAC	Plasma B	$4.56^{\ddagger}$ (±1.06)	$25.14^{\ddagger}$ (±9.41)	$5.55^{\dagger}$ (±1.48)
TOCO	Lesion	$125.3^{\#}$ (±82.50)	$212.5^{\#}$ (±184.5)	$2.42^{\#}$ (±2.31)
PLAC	Lesion	$117.0^{\#}$ (±87.73)	$164.8^{\#}$ (±95.98)	$2.62^{\#}$ (±3.24)

Results are mean (±SD). Plasma A was at recruitment, non-fasted; Plasma B was on operation day, fasted. TOCO, α-tocopherol treated patients (500 IU/day); PLAC, placebo patients; Superscripts indicate significance by Student's *t*-test: TOCO significantly different from PLAC (unpaired) $^{*}p < 0.0001$; plasma B significantly different form corresponding Plasma A (paired) $^{\ddagger}p < 0.0001$, $^{\dagger}p < 0.01$; Lesion significantly different from corresponding Plasma B (paired) $^{\#}p < 0.0001$. For other explanations see "Methods" and "Results" sections.

Plasma Lipids

Cholesterol levels were significantly lower in plasma B than plasma A, in both the TOCO and PLAC patient groups (Table I), showing declines of 16.7 and 15.2%, respectively in the mean levels. There was no significant difference between the TOCO and PLAC groups for cholesterol levels in plasma B. Considering the 4 patient sub-groups, the only significant difference in cholesterol levels in plasma B was between STTOC (3.993 ± 0.919 mM) and NSPLAC (4.743 ± 1.066 mM, $p = 0.0194$).

Fatty acids behaved commensurately with cholesterol, exhibiting lower concentrations in plasma B than plasma A, in both TOCO (total fatty acids 7.72 ± 2.93 mmol/l in A, 6.53 ± 2.24 mmol/l in B, i.e. a decrease of 15.4%) and PLAC (total fatty acids 8.06 ± 2.86 mmol/l in A, 6.83 ± 1.97 mmol/l in B, i.e. a decrease of 15.3%). There was no significant difference between TOCO and PLAC for total fatty acids in Plasma B. For the individual fatty acids, expressed as mmol/l, decreases were significant for 16:1, 16:0, 18:2, 18:1 and 18:0, but not for 20:4 and 22:6 for TOCO or 16:1 and 22:6 for PLAC, and there was no significant difference between TOCO and PLAC in plasma B for any of the individual fatty acids. When individual fatty acid levels were expressed per mol plasma cholesterol, or as a percentage of total fatty acids, there was negligible difference between plasma A and plasma B, for TOCO and PLAC, or between TOCO and PLAC in plasma B. There were no significant differences between the 4 patient sub-groups for levels of any of the individual fatty acids (mmol/l) in plasma B.

Plasma and LDL-associated α-tocopherol

Plasma α-tocopherol levels were significantly higher in plasma B than in A in the TOCO group (Table I). Contrastingly, plasma α-tocopherol levels in the PLAC group were slightly (yet significantly) lower in plasma B than in A. Plasma B α-tocopherol was significantly higher in the TOCO than the PLAC group. All these findings were true whether α-tocopherol levels were expressed as mmol/l plasma or mmol/mol cholesterol.

Considering the 4 patient sub-groups, α-tocopherol levels in plasma B were significantly higher in NSTOC (37.1 ± 13.0 μM) than in NSPLAC

TABLE II LDL oxidisability and α-tocopherol content

Patient treatment group	LDL source plasma	Lag phase (min)	Slope (AU/h)	Peak (AU)	α-Tocopherol in LDL (mmol/mol LDL cholesterol)
TOCO	Plasma A	93.91 (±17.25)	0.596 (±0.107)	0.847 (±0.071)	3.69 (±0.65)
PLAC	Plasma A	97.83 (±15.55)	0.592 (±0.090)	0.848 (±0.066)	3.68 (±0.67)
TOCO	Plasma B	$115.3^{*\ddagger}$ (±28.23)	$0.570^{*\P}$ (±0.115)	$0.827^{\dagger}$ (±0.069)	$5.65^{***\ddagger}$ (±1.46)
PLAC	Plasma B	$104.4^{\dagger\dagger}$ (±15.70)	$0.626^{\dagger}$ (±0.108)	$0.835^{\P}$ (±0.064)	$4.03^{\dagger\dagger}$ (±0.83)
	Calculation	Delta lag phase (min)	Delta slope (AU/h)	Delta peak (AU)	Delta α-tocopherol (mmol/mol LDL cholesterol)
TOCO	B minus A	21.39^{**} (±23.43)	-0.026^{**} (±0.076)	−0.020 (±0.049)	1.96^{***} (±1.43)
PLAC	B minus A	6.603 (±12.62)	0.034 (±0.078)	−0.013 (±0.037)	0.35 (±0.65)

LDL oxidisability is measured by the diene conjugation assay (absorbance 234 nm). Lag phase is the delay before onset of propagation of oxidation, slope is the maximum rate of propagation of oxidation, and peak is the maximum accumulation of conjugated dienes. For further details see "Methods" and "Results" sections. Superscripts indicate significance by Student's *t*-test: TOCO significantly different from PLAC (unpaired) $^{***}p < 0.0001$, $^{*}p = 0.0002$, $^{*}p < 0.02$; Plasma B LDL significantly different from corresponding Plasma A LDL (paired) $^{\ddagger}p < 0.0001$, $^{\dagger\dagger}p < 0.001$, $^{\dagger}p < 0.01$, $^{\P}p < 0.02$. For other explanations see footnote to Table I.

(25.8 ± 10.0 μM, $p < 0.0001$). Likewise, STTOC (42.0 ± 15.8 μM) was significantly higher than STPLAC (23.6 ± 7.83 μM, $p < 0.0001$). The only other significant differences for α-tocopherol levels (μM) in plasma B were between STTOC and NSPLAC ($p < 0.0001$) and between NSTOC and STPLAC ($p = 0.0004$). When plasma α-tocopherol levels were expressed relative to cholesterol, NSTOC (8.56 ± 3.21 mmol/mol cholesterol) was significantly higher than NSPLAC (5.45 ± 1.50 mmol/mol cholesterol, $p < 0.0001$), whilst STTOC (10.51 ± 4.05 mmol/mol cholesterol) was significantly higher than STPLAC (5.81 ± 1.44 mmol/mol cholesterol, $p < 0.0001$). The only other significant differences for plasma α-tocopherol (mmol/mol cholesterol) were between STTOC and NSPLAC ($p < 0.0001$), between NSTOC and STPLAC ($p = 0.0012$) and between NSTOC and STTOC ($p = 0.0173$).

Compared with plasma A LDL, there was a 53 and 9% increase in α-tocopherol in plasma B LDL in the TOCO and PLAC groups respectively (Table II). The mean α-tocopherol level in plasma B LDL was 40% higher in the TOCO than in the PLAC group. Clearly, oral α-tocopherol supplementation significantly increases both total plasma α-tocopherol and LDL-associated α-tocopherol in the plasma.

Consideration of the 4 patient sub-groups also revealed significant increases in α-tocopherol within plasma LDL as a result of supplementation. α-Tocopherol in LDL from plasma B was significantly higher in the NSTOC group than in the NSPLAC group (5.52 ± 1.48 and 3.92 ± 0.69 mmol/mol LDL cholesterol respectively, $p < 0.0001$), and likewise significantly higher in STTOC than in STPLAC (5.97 ± 1.43 and 4.28 ± 1.07 mmol/mol LDL cholesterol respectively, $p = 0.0002$). The only other significant differences for α-tocopherol in plasma B LDL were between STTOC and NSPLAC ($P < 0.0001$) and between NSTOC and STPLAC ($p = 0.0010$). Delta α-tocopherol in LDL in NSTOC was significantly higher than in NSPLAC (1.90 ± 1.40 and 0.32 ± 0.42 mmol/mol LDL cholesterol respectively, $p < 0.0001$), and likewise significantly higher in STTOC than in STPLAC (2.11 ± 1.54 and 0.42 ± 1.02 mmol/mol LDL cholesterol respectively, $p < 0.0001$). The only other significant differences for delta α-tocopherol in LDL were between STTOC and NSPLAC ($p < 0.0001$) and between NSTOC and STPLAC ($p < 0.0001$). There was no significant difference between NSTOC and STTOC or between NSPLAC and STPLAC for either α-tocopherol in plasma B LDL or for delta α-tocopherol in LDL.

LDL Oxidisability *Ex Vivo*

These results are presented in Table II. Compared with plasma A LDL, mean lag phase (the delay before the onset of propagation of oxidation) was significantly increased by 23 and 7% in plasma B LDL for the TOCO and PLAC groups, respectively, and lag phase for plasma B LDL was significantly longer in the TOCO than in the PLAC group. The mean increase (delta, i.e. B minus A) in lag phase was also significantly greater for TOCO than for PLAC.

Slope (the maximum rate of propagation of oxidation) for the TOCO group was significantly lower for plasma B LDL than for plasma A LDL, whereas for PLAC the reverse was true (i.e. B higher than A). Slope for Plasma B LDL was significantly lower in the TOCO group than in PLAC. Delta slope (B minus A) was a negative value for the TOCO group but positive for PLAC, with delta slope for TOCO being significantly lower than for PLAC.

Peak (the maximum level of conjugated dienes) diminished slightly but significantly for both the TOCO and PLAC groups for Plasma B LDL compared with Plasma A LDL. There was no significant difference between TOCO and PLAC for peak for Plasma B LDL, or for delta peak (B minus A).

Delta lag phase correlated with delta α-tocopherol, thus the decrease in oxidisability of the LDL was commensurate with the increase in the LDL-associated α-tocopherol, whether considering data from all patients (i.e. TOCO and PLAC pooled; Fig. 1a) or TOCO patients only (Fig. 1b). In both these cases the correlation was linear and highly significant (Fig. 1). For the PLAC group the correlation was weaker and poorly linear ($r^2 = 0.035$) yet still statistically significant (tau = 0.220, $p = 0.0244$ by Kendall's rank correlation). Taken together these results are consistent with the expectation that oral α-tocopherol therapy inhibits oxidation of plasma LDL.

Regarding the 4 patient sub-groups, LDL oxidisability results were as follows. α-tocopherol supplementation significantly decreased the oxidisability of the LDL *ex vivo* in NSTOC compared with NSPLAC. For lag phase (plasma B LDL) the only significant difference was between NSTOC (117.0 ± 28.6 min) and NSPLAC (103.4 ± 15.6 min, $p = 0.0145$). For delta lag, the only significant differences were between NSTOC (23.5 ± 22.4 min) and NSPLAC (6.56 ± 10.92 min, $p = 0.0003$), and between NSTOC and STPLAC (6.71 ± 16.4 min, $p = 0.0049$). For NSTOC there was a significant correlation between delta lag and delta α-tocopherol ($p < 0.0001$). For slope, the only significant difference was between NSTOC (0.562 ± 0.108 AU/h) and NSPLAC (0.628 ± 0.107 AU/h, $p = 0.0146$). For delta slope, the only significant differences were between NSTOC (−0.040 ± 0.070 AU/h) and NSPLAC (0.023 ± 0.066 AU/h, $p = 0.0007$), between NSTOC and STTOC (0.007 ± 0.083 AU/h, $p = 0.0472$) and between NSTOC and STPLAC (0.059 ± 0.099 AU/h, $p < 0.0001$). There were no significant differences between the groups STTOC

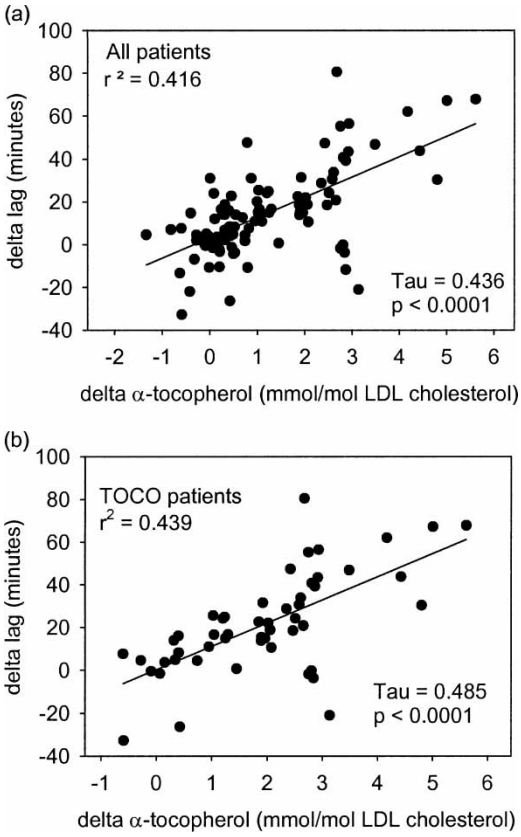

FIGURE 1 Delta lag phase (minutes) for LDL oxidation *ex vivo* plotted against delta α-tocopherol (mmol/mol LDL cholesterol). (a) All patients in the study, irrespective of treatment, (b) the α-tocopherol-treated (TOCO) group of patients. Delta designates difference, i.e. plasma B LDL minus plasma A LDL.

and STPLAC, or between STPLAC and NSPLAC in terms of lag, delta lag, slope or delta slope. There were no significant differences between any of the 4 patient sub-groups when peak or delta peak were considered.

Relative Changes of LDL and HDL Levels in Plasma

On agarose electrophoresis gels, both LDL and HDL bands were significantly weaker in plasma B than in plasma A, for both TOCO and PLAC patient groups ($p < 0.0001$). For LDL, the mean decreases were 25.2 ± 25.4 and 26.1 ± 34.5% for the TOCO and PLAC groups, respectively, whilst HDL decreased by 24.7 ± 26.3 and 20.6 ± 24.9% for TOCO and PLAC, respectively. There was no significant difference between TOCO and PLAC for these percentage decreases, likewise between the 4 patient sub-groups. There was no significant difference between plasmas A and B for LDL/HDL ratio, for TOCO (5.20 ± 2.79 in A and 5.42 ± 3.29 in B), or for PLAC (4.50 ± 1.88 in A and 4.39 ± 2.19 in B). In plasma B, there was no significant difference between TOCO and PLAC, for LDL/HDL ratio. For the 4 patient sub-groups the only significant difference for LDL/HDL ratio in plasma B was between NSTOC (5.40 ± 3.44) and STPLAC (3.60 ± 1.83, $p = 0.0393$).

Lesion α-tocopherol

There was no significant difference between the TOCO and PLAC groups for lesion α-tocopherol levels (expressed as nmol/g lesion wet weight or as mmol/mol cholesterol), or for lesion cholesterol levels (Table I). Alpha-tocopherol levels, not standardised for cholesterol, were significantly higher in lesions than in plasma when α-tocopherol was expressed relative to wet weight, for both TOCO and PLAC groups. For comparison with lesions, 1 μmol α-tocopherol/l plasma equals 1 nmol α-tocopherol/g wet weight plasma to a close approximation, since the mean specific gravity of plasma is 1.0269 (95% range 1.0251–1.0287).[18] However, when α-tocopherol levels were cholesterol-standardised (i.e. expressed as mmol/mol cholesterol), levels in lesions were significantly lower than in plasma, for both TOCO and PLAC groups.

Lesion α-tocopherol levels (nmol/g wet weight) correlated significantly with those in plasma (μmol/l) when all patients were considered (i.e. TOCO and PLAC groups pooled), by Kendall's rank correlation (tau = 0.155, $p = 0.0193$). However, this relationship became insignificant when lesion and plasma α-tocopherol levels were cholesterol-standardised, (i.e. expressed as mmol/mol cholesterol), or when the TOCO or PLAC groups or any of the 4 patient sub-groups was considered separately and α-tocopherol expressed as nmol/g wet weight (or μmol/l) or as mmol/mol cholesterol.

Thus, although oral α-tocopherol supplementation increased LDL-associated α-tocopherol concentrations in the circulation, this was not reflected in increases in cholesterol-standardised α-tocopherol concentrations within established atherosclerotic plaques.

Relationship in Lesions Between α-tocopherol and 7β-hydroxycholesterol

There was an inverse correlation between levels of α-tocopherol and 7β-hydroxycholesterol in lesions, when both were standardised for cholesterol (i.e. expressed as mmol/mol cholesterol). This was highly significant whether considering all patients (i.e. TOCO and PLAC groups pooled; Fig. 2a) or the TOCO group only (Fig. 2b), whilst for the PLAC group it did not achieve significance (tau = −0.181, $p = 0.0606$).

Regarding the 4 patient sub-groups, there was a clear inverse trend (tau = −0.38, $p = 0.0009$) between levels of α-tocopherol and 7β-OH-CHOL (when both were expressed relative to cholesterol) in lesions from NSTOC patients, and this inverse

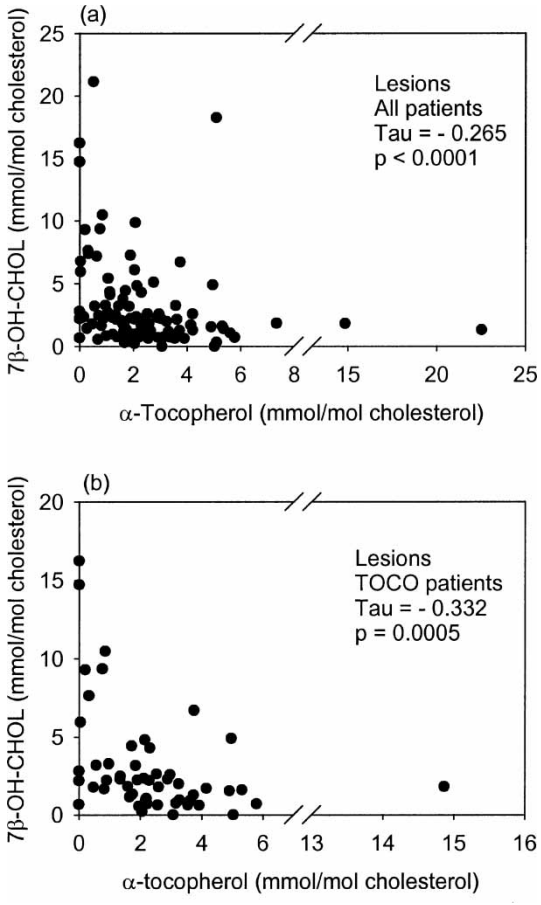

FIGURE 2 Levels of 7β-hydroxycholesterol (7β-OH-CHOL) (mmol/mol cholesterol) in lesions plotted against levels of α-tocopherol (mmol/mol cholesterol). (a) All patients in the study irrespective of treatment, (b) the α-tocopherol-treated (TOCO) group of patients.

trend was also significant (tau $= -0.238$, $p = 0.0379$) when levels were expressed relative to wet weight. There was no corresponding trend ($p > 0.1$) between α-tocopherol and 7β-OH-CHOL (when both were expressed relative to cholesterol or relative to wet weight) in lesions from NSPLAC, STTOC or STPLAC patients.

There was no significant difference between TOCO and PLAC for mean levels of 7β-hydroxycholesterol in lesions (TOCO group: 437 ± 707 nmol/g wet weight, 3.09 ± 3.44 mmol/mol cholesterol; PLAC group: 420 ± 724 nmol/g wet weight, 3.21 ± 4.02 mmol/mol cholesterol). Between the 4 patient sub-groups, there were no statistically significant differences for levels of 7β-hydroxycholesterol (expressed relative to wet weight or relative to cholesterol) in lesions, or for levels of cholesterol in lesions.

DISCUSSION

The basic assumption underlying clinical trials of oral antioxidant vitamins has been that they will exert a biochemical effect, not only within plasma but also more importantly within atherosclerotic plaques where the majority of lipid peroxidation appears to take place. Our aim was to test this assumption.

Evidence for Antioxidant Effects of Oral α-tocopherol in Plasma LDL and in Lesions

We showed that oral α-tocopherol supplementation clearly increased α-tocopherol concentrations in plasma and within plasma LDL, and decreased the susceptibility of LDL to *ex vivo* oxidation, consistent with previous studies.[17,19,20] We also showed that lesion α-tocopherol concentrations (expressed independently of lesion cholesterol concentrations) correlated positively with plasma α-tocopherol levels, a relationship generally assumed, but never before demonstrated in a human supplementation study. Furthermore, we found an inverse correlation between cholesterol-standardised lesion concentrations of α-tocopherol and 7β-hydroxycholesterol, a free radical oxidation product of cholesterol. Thus, in addition to protecting circulating LDL from oxidation, our results suggest that α-tocopherol also inhibits LDL oxidation within established atherosclerotic lesions Although oral α-tocopherol supplementation significantly increased circulating LDL-associated α-tocopherol by some 50%, there was no increase in plaque cholesterol-standardised α-tocopherol mean concentration.

Significance of Plasma α-tocopherol Levels

At recruitment (prior to supplementation) the mean plasma levels of α-tocopherol in our patients (plasma A in Table I) were similar to that of the CHAOS trial patients at recruitment (mean 34.2 μM),[8] and are at the high end of the "normal" range (e.g. 26.58 ± 8.01 μM, $n = 28$).[21] The explanation is unknown, but might be due to these patients being mostly elderly, with advanced atherosclerosis. Age-related increases in α-tocopherol levels are reported in human plasma[22] and in rats (plasma and tissues, including normal aorta).[23] This phenomenon may represent a compensatory mechanism that attempts to counterbalance age-associated oxidative stress.[23] When standardised for cholesterol, the plasma α-tocopherol levels in our patients at recruitment (plasma A in Table I) remained greater than those of "normal" volunteers (5.03 ± 1.26 mmol/mol cholesterol).[21] By operation day, the mean plasma α-tocopherol level in the placebo patients (plasma B in Table I) was close to "normal", expressed both as absolute concentration (μM) and as mmol/mol cholesterol.

The measured changes in plasma α-tocopherol levels induced by dietary supplementation in

the present study were modest. However, plasma levels of α-tocopherol peak at around 11–12 h after ingestion,[24–27] and our patients were fasted on their operation day. Therefore, the difference in plasma concentrations of α-tocopherol between operation day (B, fasting) compared with at recruitment (A, non-fasting), probably underestimates the increase in α-tocopherol levels achieved in those patients taking α-tocopherol.

Absolute tocopherol levels are widely believed to be of much less functional significance than those related to lipid concentrations, and we found substantial changes in LDL α-tocopherol in the actively treated patients. α-Tocopherol is incorporated into several different lipoprotein fractions and since LDL α-tocopherol levels are more stable than in less dense lipoprotein fractions,[24,25] they are more likely to reflect the "steady state" in patients taking chronic oral supplements. Furthermore, the measured increase in LDL-associated α-tocopherol occurred on a background of lipid-lowering, including LDL lowering, in both groups between recruitment and operation, attributable to dietary advice at recruitment and to the initiation of statin therapy in a small, but equal, proportion of both groups of patients.

The lowering of HDL as well as LDL in both TOCO and PLAC groups, with no change in the LDL/HDL ratio might be partly due to fasting on operation day. Another contributing factor might be insufficient change in the pattern of dietary fatty acids consumed between recruitment and operation day, consistent with the similarity in the plasma fatty acid profiles (i.e. individual fatty acids as percentages of total fatty acids) at recruitment and on operation day. Low-lipid, high-carbohydrate diets can lower HDL as much as LDL, unless there is significant substitution of saturated fatty acids by unsaturated fatty acids.[28]

Significance of α-tocopherol Levels in Lesions

The positive, significant correlation between plasma and lesion α-tocopherol in the present study provides the first direct evidence that plasma levels of antioxidants influence their levels in advanced atherosclerotic lesions. However, whilst oral α-tocopherol treatment caused a substantial (~50%) increase in the lipid-standardised α-tocopherol in plasma (i.e. both total plasma cholesterol-standardised α-tocopherol and plasma LDL-associated α-tocopherol), with an associated reduction in LDL oxidisability, there was no corresponding significant increase in mean cholesterol-standardised α-tocopherol concentration in lesions. This observation, together with the finding that in lesions, when compared to plasma, α-tocopherol was depleted relative to cholesterol (discussed below), might help rationalise the disappointing results of recent clinical trials of the effects of dietary antioxidant vitamins on cardiovascular events.

The relatively low level of cholesterol-standardised α-tocopherol in lesions compared to plasma might be due to some loss of α-tocopherol as cholesterol is concentrated in the plaque, and/or to the relatively short duration of our study, necessitated by the clinical imperative to operate within a reasonable period of presentation. Alternatively, it might reflect increased consumption of α-tocopherol by oxidative activity within the lesion. Since 7β-OH-CHOL is a product of free radical oxidation of cholesterol,[29] the observed inverse correlation between α-tocopherol and 7β-OH-CHOL is consistent with the extensive *in vitro* evidence that α-tocopherol acts as a radical scavenger and suggests a similar role within atherosclerotic lesions *in vivo*.

Effects of Statins

The present study was not designed at the outset to test the effects of statins. However, their prescription to a minority of patients during the study enabled us to sub-group the patients' data in an attempt to elucidate their effects, if any. In the event, this revealed little evidence for effects of statins per se, although they appeared to blur certain effects of α-tocopherol. Notably, the inverse correlation in lesions between α-tocopherol and 7β-hydroxycholesterol, which was significant in the NSTOC group, was insignificant in the STTOC group. Statins or their metabolites can act as radical scavengers,[30–32] and so might blur the inverse relationship between α-tocopherol and 7β-hydroxycholesterol, which is a product of free radical oxidation. Moreover, whilst LDL from NSTOC was significantly less oxidisable than was LDL from NSPLAC, oxidisability of LDL from STTOC was not significantly different to that of LDL from STPLAC, consistent with an antioxidant effect of statins. However, there was no significant difference between STPLAC and NSPLAC for LDL oxidisability, arguing against a major antioxidant effect of statins.

The important conclusion to be drawn from subsidiary consideration of statin status (i.e. the 4 patient sub-groups) is that the main findings of the study based on α-tocopherol supplementation (i.e. the TOCO and PLAC groups, which both contain a small but equal proportion of patients receiving statins) still stand valid when statin patients are discounted. The evidence for antioxidant effects of α-tocopherol supplementation, revealed by this study within plasma LDL and within lesions, are thus clearly not just artefacts of statin treatment, and grouping patients by "intention to treat" (i.e. TOCO or PLAC) is thereby justified.

Choice of α-tocopherol Regimen

This study's results suggest that the dose of antioxidants used in several clinical trials might have been insufficient to prevent cholesterol oxidation within advanced atherosclerotic lesions. Notably, in CHAOS, a study in which α-tocopherol appeared to protect against coronary events, just over half of the actively treated population received 800 IU/d α-tocopherol, a substantially higher dose than in subsequent studies that failed to demonstrate an effect.[8–11] For the present study, we chose a moderately high (500 IU/d) dose of α-tocopherol, intermediate between the two dose levels (400 and 800 IU/d) used in CHAOS.[8] The latter was not a dose-response study, the lower dose being adopted in approximately half of the actively treated patients because of supply constraints. The issue of dose-response was outside the scope of the present study, as it would have required many more patients.

The CHAOS trial used the natural (RRR) form of α-tocopherol.[8] The synthetic form of α-tocopherol (all-rac), used in the present study because of supply constraints, is considered to be somewhat less bioavailable than the natural RRR-form.[33,34] Despite this, our oral supplementation regimen resulted in statistically significant increases in α-tocopherol levels in plasma, and, more markedly, within plasma LDL, and significantly protected the LDL against *ex vivo* oxidation, criteria by which other studies found oral supplementation with all-rac or RRR forms of α-tocopherol to be equally effective.[19,20] Further supplementation studies are needed to address the important issues of dose and stereochemistry of α-tocopherol.

CONCLUSIONS

In summary, this study has demonstrated an inverse correlation between plaque α-tocopherol concentrations and cholesterol oxidation and has shown that approximately 3 months' ingestion of 500 IU α-tocopherol/day increases the resistance of plasma LDL to oxidation, but does not significantly elevate the cholesterol-standardised α-tocopherol mean concentration in established atherosclerotic plaques. These findings support the notion that antioxidant vitamins can protect against lipid oxidation in atherosclerosis. This idea is supported by findings in apoE-deficient mice, in which vitamin E lowered oxidative stress *in vivo* and reduced atherosclerotic lesion formation.[35] Vitamin E also reduced progression of atherosclerosis in LDL receptor-deficient mice with established vascular lesions.[36] Very recently it was reported that supplementation with vitamin E (136 IU) plus vitamin C (250 mg), twice daily for 6 years, significantly reduced carotid atherosclerosis progression, determined ultrasonographically, in hypercholesterolemic men.[37] More detailed investigation of the pharmacodynamics of oral antioxidant vitamins is needed, in case their rejection as potential therapeutic agents in human atherosclerosis might be premature.

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