α-Tocopherol protects against diet induced atherosclerosis in New Zealand white rabbits

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Abstract   In this study, we asked the question "does α-tocopherol supplementation prevent an increase in total plasma cholesterol (TPC) concentration and reduce the deposition of cholesterol in arterial plaques of rabbits fed atherogenic diets?" Isocaloric diets containing 0.1% cholesterol to induce atherosclerosis were enriched in one of three fats: saturated fats (SAT), monounsaturated fats (MONO), or n-6 polyunsaturated fats (POLY). Half of each of the three diets were supplemented with 2,500 IU α-tocopherol/kg-diet. Unsupplemented diets contained 25 IU α-tocopherol/kg-diet. Rabbits supplemented with α-tocopherol had plasma α-tocopherol concentrations 10-fold higher and an average TPC concentration 31% lower, $P = 0.017$, than rabbits fed unsupplemented diets. Among the three fat-fed groups, the difference was greatest for the POLY fat fed group (54%, $P = 0.041$). POLY fat-fed rabbits without α-tocopherol supplementation had plasma HDL cholesterol concentrations that were less than half that of rabbits fed other fats, $P \leq 0.0001$. In general, differences in mean esterified artery cholesterol concentrations among the three fat-fed groups, with and without α-tocopherol supplementation, paralleled differences in TPC concentration among the groups. This study suggests that for rabbits fed high pharmacological doses of α-tocopherol, atherosclerosis can be diminished in situations where the plasma cholesterol concentrations are also significantly lower.—Schwenke, D. C., L. L. Rudel, M. G. Sorci-Thomas, and M. J. Thomas. **α-Tocopherol protects against diet induced atherosclerosis in New Zealand white rabbits.** *J. Lipid Res.* 2002. **43:** 1927–1938.

Supplementary key words   α-tocopherol • fatty acid • polyunsaturated fatty acid • saturated fatty acid • monounsaturated fatty acid • high density lipoprotein • lipoprotein

Oxidation of LDL is thought to promote atherosclerosis and cardiovascular disease in humans (1). It should follow then that α-tocopherol and saturated fats would retard in vitro oxidation of LDL (2–9). Thus, one might expect α-tocopherol and a diet rich in saturated fats to reduce atherosclerosis by retarding the in vivo oxidation of LDL (10–17). However, in healthy subjects α-tocopherol did not reduce markers of oxidative stress like urinary F_2-isoprostanes (18), and in some cases actually increased the plasma concentration of F_2-isoprostanes (19).

It is also well known that in humans and animals, high levels of dietary polyunsaturated fatty acids (PUFAs) reduce HDL cholesterol (HDL-C) (13, 20, 21), a process that would further increase the risk of atherosclerosis. Therefore, an increased concentration of linoleate in the plasma lipids would be expected to correlate with an increased risk of coronary heart disease (CHD) (22–25). However, men having a higher concentration of linoleate in plasma or adipose lipids are reported to have a lower risk of CHD (26, 27). Both non-human primates and rabbits have been shown to develop less atherosclerosis when fed polyunsaturated fat-rich diets that increase the polyunsaturated fat content of plasma lipid (12, 28–30). Therefore, the relationships between the fatty acid composition of the diet, plasma lipoprotein concentrations, and the risk of atherosclerosis and CHD are not clear cut and the risk of disease may depend on the interaction between several metabolic variables.

Several reviews of the literature have suggested that α-tocopherol or other antioxidants may reduce atherosclerosis or CHD (14, 31–34). High levels of dietary α-tocopherol have been associated with reduced CHD in several population studies of men and women (35–38). One clinical trial of cholesterol lowering also observed reduced atherosclerosis progression in men who chose to consume high

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Abbreviations: BHT, butylated hydroxytoluene; DTPA, diethylenetriaminepentaacetic acid; MONO, diet group fed a diet enriched with monounsaturated fats; POLY, diet group fed a diet enriched with polyunsaturated fats; SAT, diet group fed a diet enriched with saturated fats.
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levels of α-tocopherol supplements (39). Based on such suggestive observations, several randomized clinical trials have been conducted to determine whether supplementation with 50–800 IU α-tocopherol reduces cardiovascular disease (CVD) (32, 40–43) or risk factors for CVD (44–48). Stephens et al. (32) reported that 400–800 IU α-tocopherol supplementation reduced the risk of nonfatal myocardial infarction in patients with proven coronary atherosclerosis. No change in fatal and non-fatal myocardial infarctions were reported in high risk patients given α-tocopherol supplements (17, 42).

Several randomized, placebo-controlled double blind studies have shown that modest α-tocopherol supplementation slightly increased HDL-C in hypercholesterolemic patients (49) and in patients on hemodialysis (50). A single study using parallel groups with no supplement or supplemented with 100 IU α-tocopherol/day detected a small decrease in HDL-C after supplementation (51). Other randomized, placebo controlled double-blind studies reported no significant change in plasma cholesterol concentration (43–48). The pro-and anti-oxidant roles proposed for α-tocopherol taken together with the results from clinical trials have generated some controversy regarding the clinical value of α-tocopherol supplements (14–17, 52, 53). Therefore, the role of α-tocopherol needs to be clarified, so that the segment of the population that might benefit from α-tocopherol supplements can be identified.

α-Tocopherol has other physiologic roles in addition to that of inhibiting free radical autoxidation. Rabbits fed hypercholesterolemic diets have had an improved arterial relaxant response when supplemented with doses of α-tocopherol on the order of 1,000 IU/kg diet (54–58). Other studies have shown that α-tocopherol modulated phosphorylation of PKC in the rabbit artery (59–61) and that dietary supplements were associated with lower activity of hepatic HMG-CoA reductase and acyl-CoA cholesterol acyltransferase in rats (62). Recent studies in humans (2, 63) and hamsters (64) suggested that α-tocopherol increased the activity of cholesterol ester transfer protein (CETP), a protein that promotes exchange of cholesterol and triglyceride among lipoproteins (65). Studies of naturally occurring CETP deficiency in humans (66), of transgenic mice over-expressing human CETP (67, 68), and biological manipulation of CETP concentration (69) have demonstrated a reciprocal relationship between CETP activity and plasma HDL concentration.

Several studies reported that animals fed a cholesterol-rich diet supplemented with α-tocopherol have a lower TPC than animals consuming low levels of dietary α-tocopherol: male New Zealand white rabbits (57–59, 61, 70, 71), Watanabe heritable hyperlipidemic rabbits (4), Wistar rats (72), and apolipoprotein E (apoE) deficient mice (10, 43, 73). Two studies reported no change in TPC concentrations in both male and female New Zealand white rabbits (54, 74). In the absence of added cholesterol, α-tocopherol did not lower the TPC concentration (1, 75–77). α-Tocopherol supplementation did not prevent lesion development in injured rabbit arteries but did reduce lipid peroxidation (78).

However, no studies have explored combinations of α-tocopherol and dietary fat. In this study, we sought to determine the effects of dietary fat type (saturated, mono-unsaturated, n–6 polyunsaturated) and α-tocopherol on the plasma cholesterol concentration and atherosclerosis. We conducted these studies in rabbits fed diets containing one of the three fats with 0.1% cholesterol included in the diet to induce hypercholesterolemia. These three fat types are the main constituents of Western diets and their roles in the development of atherosclerosis have been studied in animal models (28, 30). To maximize the pharmacologic response in rabbits, the average daily supplement of α-tocopherol used in this study, about 45 IU/kg-body weight, was approximately 4-times higher than the maximum daily dose given to volunteers participating in some clinical trials. We found that a consequence of α-tocopherol supplements was a lower cholesterol concentration and less cholesterol deposition in atherosclerotic plaques, with the greatest effect when dietary fat was polyunsaturated.

MATERIALS AND METHODS

Diets

In this study, we used six different diet-groups. All diets contained 0.1% cholesterol to induce hypercholesterolemia, one of three different dietary fats (saturated, monounsaturated, n-6 polyunsaturated), and α-tocopherol at two different levels. These diets were modifications of a hypercholesterolemic diet used in a previous study (79). We reduced the basal α-tocopherol content to 25 IU/kg and used the following test fats: saturated fat (SAT) supplied as a 50:50 mixture (kcal/kcal) of beef tallow and dairy butter, monounsaturated fat (MONO) supplied as high oleate sunflower oil (Trisun, high oleate), and n-6 polyunsaturated fat (POLY) supplied as high linoleate sunflower oil (Trisun, high linoleate). Both sunflower oils were stripped to remove endogenous tocopherols. The α-tocopherol supplement used in this study was d,l α-tocopherol acetate (2,500 IU/kg diet, or about 125 IU/rabbit/day). The caloric distribution of the major dietary components was 22.1% protein, 53.2% carbohydrate, and 24.7% fat. The diets were prepared by Research Diets, Inc. (New New Brunswick, NJ). We included one group of rabbits fed Pro-Lab Hi-Fiber Rabbit chow (PMI Nutrition International, Inc) as a control.

Rabbits

Young sexually mature male New Zealand white rabbits were obtained from Robinson Services, Inc. (Winston-Salem, NC). Rabbits were acclimated to the animal facility for 1-week during which time they were maintained on a standard cholesterol-free rabbit chow. After acclimation to the animal facility, rabbits were assigned to one of the six fat-fed diet groups or to a chow-fed group. The fat-fed groups, six animals per group, were fed diets that contained either high (2,500 IU/kg-diet) or low levels (25 IU/kg-diet) of α-tocopherol. Rabbits were fed the above diets for twelve weeks, and then they were terminated for collection of tissues for analyses. Among the original 42 rabbits in this study, data from three rabbits were excluded from analysis. The excluded rabbits included one sick rabbit each from SAT and POLY fat-fed groups. One animal in the POLY + high α-tocopherol (POLY+VE) was found to be a female. We measured plasma parameters in 39 rabbits: 5 SAT, 6 SAT+VE, 6 MONO, 6

MONO+VE, 5 POLY, 5 POLY+VE, and 6 CHOW. Arteries from 38 animals were analyzed. One artery segment from the SAT+VE group had not been immediately frozen and was excluded from the artery analyses. The Wake Forest University School of Medicine Animal Care and Use Committee approved all procedures.

Plasma and lipoprotein lipids

Blood samples were collected from the ear veins of rabbits before diet treatment began while rabbits were consuming rabbit chow, and then every 2 weeks during treatment until the end of the study at 12 weeks. After an overnight fast, blood samples were collected into disodium EDTA (4 mmol/l final concentration). Plasma was obtained after low speed centrifugation and supplemented with butylated hydroxytoluene (20 μmol/l final concentration). TPC and triglyceride concentrations were determined by enzymatic methods in the Lipid Analytical Laboratory of Wake Forest University School of Medicine, Winston-Salem, NC. HDL-C was determined in supernatants obtained after precipitation with heparin-manganese (80).

Artery preparation

We selected for study the proximal one quarter of the descending thoracic aorta designated t1, an arterial segment bounded proximally by the ductus scar and distally by the celiac orifice. The susceptibility to atherosclerosis for this arterial segment, both in terms of development of sudanophilic atherosclerotic lesions (81, 82) and arterial cholesterol accumulation (83) is intermediate between the aortic arch, the most atherosclerosis-susceptible arterial region (84, 85) and the distal aorta. The rabbits were euthanized with sodium pentobarbital at 100 mg/kg body weight delivered IV. The body cavity was opened and the aorta extending to the iliac bifurcation was excised. The artery was washed with cold PBS buffer containing 0.1 mM diethylenetriaminepentaacetic acid (DTPA), 80 μM butylated hydroxytoluene (BHT), and 0.1 mM triphenylphosphine. Residual adventia was removed. The samples were then quickly frozen in liquid nitrogen and stored at −70°C until analysis.

Extraction of lipophilic components

Artery tissue, 50 mg wet weight of the t1 segment of the throacic aorta, was frozen in liquid nitrogen and then ground with a mortar and pestle. Lipids were extracted using isopropanol-hexane (86) that contain BHT, triphenylphosphine, and diethylenetriaminepentaacetic acid. The extracts were dried in a stream of argon gas then diluted with 2 ml methanol. Lipid extracts were stored at −70°C under argon before analysis.

Quantitation of α-tocopherol

HPLC was used to measure α-tocopherol. Separation was achieved on a 150 mm × 2 mm C_{18} column packed with 3 micron diameter particles. The eluant was 95% methanol, 5% 1 M acetate at pH 5.5 at a flow rate 0.41 ml/min from a Hewlett Packard model 1090 HPLC. δ-Tocopherol was used as the internal standard. An ESA CoulArray detector was used to detect α-tocopherol. The standard concentration curve was constructed from 40 pg to 200 pg injected. However, where necessary we were able to quantify α-tocopherol at 1 pg per injection. The purity of the tocopherols was ascertained by checking the concentration by uv spectroscopy using $\varepsilon = 3{,}467 \text{ mol}^{-1}\text{cm}^{-1}$ at 292 nm.

Fatty acid composition

The method is based on that of Metcalfe et al. (87). Pentadecanoic acid was added to the lipid extract as the internal standard. The sample plus standard were dried in a stream of nitrogen gas, 0.1 ml of 0.5 N NaOH in methanol was added, the tube was purged with nitrogen gas, then heated to 100°C for 5 min.

After cooling, 0.1 ml 14% BF_3 in methanol (Pierce) was added to each tube. The tubes were heated to 100°C for 5 min then cooled to room temperature. Hexane (500 μl) and saturated aqueous solution of NaCl (200 μl) were added and the layers were separated. After drying the hexane layer with anhydrous Na_2SO_4, the hexane phase was transferred to a separate tube, dried in a stream of nitrogen, the residue dissolved in isooctane, and then analyzed on a Hewlett Packard model 5,890 gas chromatograph. Separation was accomplished using a 30 m × 0.25 mm diameter DB-225 WCOT column (J and W Scientific) with a 0.25 micron coating.

Cholesterol analysis

The method is based on the report of Haeffner and Hoffmann (88). Sigmasterol was added to 100 μl of the lipid extract that was then further divided into two equal aliquots. The first aliquot was dried in a stream of nitrogen, taken up in undecane, and then injected into the gas chromatograph to determine the mass of free cholesterol. The second fraction was dried in a stream of nitrogen, saponified in 0.5 N ethanolic KOH for 15 min at 60°C. The tube was cooled, water was added, and the cholesterol extracted into hexane. After evaporating the hexane under nitrogen, the residue was dissolved in undecane and injected into the gas chromatograph to get the total mass of cholesterol. These analyses were performed on a Hewlett Packard model 5890 gas chromatograph equipped with a 30 m × 0.32 mm diameter WCOT column coated with a 0.25 micron layer of SE-30.

Statistical methods

Data for groups of rabbits fed the hypercholesterolemic diet was analyzed by ANOVA considering the factorial nature (three levels of dietary fat saturation ×2 levels of dietary α-tocopherol) of the design. A P value of <0.05 was considered significant. Analysis of covariance (ANCOVA) was used to investigate differences among groups that were independent of differences in baseline values. No interactive effects between fat saturation and dietary α-tocopherol was found (P values for interaction always ≥0.18 and usually >0.50); therefore, this interaction was excluded from the statistical models. Post hoc analysis was conducted using the Bonferroni correction (89). When necessary to minimize variances among groups, data were transformed to logarithms. Analyses were performed using STATVIEW software and SAS 6.12 (SAS Institute, Cary, NC).

RESULTS

General

The plasma cholesterol concentrations measured for each group at the end of the acclimation period did not change significantly after 12-weeks on a chow diet. Before treatment, the plasma cholesterol concentrations and body weight did not differ significantly among the groups. There was no significant difference in body weights among the fat-fed groups after 12-weeks. However, after 12-weeks CHOW-fed animals were 9% and 6% heavier than SAT and MONO fat-fed animals, respectively, $P <$ 0.0016. The weights of the chow-fed and POLY animals were not significantly different.

Total plasma cholesterol concentration

Among the fat-fed groups the MONO group had a significantly lower TPC than the SAT group, $P = 0.0076$. The results are summarized in **Table 1**. The TPC concentra-

TABLE 1. Plasma cholesterol and α-tocopherol concentrations for rabbits fed fat-rich diets for 12 weeks

Dietary Group	N	TPC	HDL	NonHDL-C	Triglyceride	Plasma α-Tocopherol
		mg/dl				*μg/ml*
SAT	5	743 ± 170^a	35 ± 2^a	708 ± 171^a	75 ± 64^a	11.7 ± 7.4^a
SAT+VE	6	518 ± 114^a	30 ± 5^a	487 ± 115^a	56 ± 19^a	164.6 ± 48.0^b
MONO	6	408 ± 6^b	32 ± 3^a	375 ± 60^b	36 ± 12^a	6.2 ± 1.6^a
MONO+VE	6	299 ± 24^b	23 ± 3^a	276 ± 21^b	32 ± 13^a	83.7 ± 12.6^b
POLY	5	$682 \pm 131^{a,b,c}$	14 ± 2^b	$668 \pm 132^{a,b,c}$	74 ± 56^a	13.3 ± 6.4^a
POLY+VE	5	$328 \pm 64^{a,b,d}$	11 ± 2^b	$317 \pm 65^{a,b,d}$	37 ± 4^a	76.1 ± 28.8^b
CHOW	6	37 ± 4	18 ± 1	19 ± 3	46 ± 17	1.8 ± 0.4

Results for rabbits fed a chow diet for 12 weeks are included for comparison. Concentrations are given as the Mean ± SEM. ANCOVA was used Diet and VE as independent variables. VE, high α-tocopherol.

[a,b,c,d] Statistically significant differences from Bonferroni-Dunn post-hoc analysis. The values for animals fed a chow diet for 12 weeks were not significantly different from the values measured at week 0 before beginning the dietary study, and were not included in ANOVA. ANOVA for TPC and NonHDL-C with VE as the independent variable and split by diet gave a statistically significant difference between POLY and POLY+VE. These differences are indicated by [c] and [d].

tion was lower in all groups by an average of 31% when the diet was supplemented with 2,500 IU α-tocopherol/kg-diet compared with rabbits supplemented with 25 IU α-tocopherol/kg-diet, $P = 0.017$. Comparing the high and low α-tocopherol levels on the individual fat-fed groups showed that the TPC concentration of the POLY group on the high α-tocopherol supplement was 54% lower compared with the POLY group with low α-tocopherol, $P = 0.041$. **Figure 1A** shows the effect of dietary fat on TPC concentration after 12-weeks of treatment. Figure 1B shows the main effect of α-tocopherol supplement on TPC concentration after 12 weeks.

HDL-C concentration

At 12-weeks, animals in the SAT and MONO diet groups had HDL-C concentrations that were greater than those of the POLY fat-fed group, $P \leq 0.0001$. **Figure 2A** shows data for the effect of dietary fat saturation and α-tocopherol supplement on HDL-C adjusted for the pre-treatment HDL-C concentrations. When both fat satura-

tion and α-tocopherol were taken into account the effect of α-tocopherol barely reached statistical significance, $P = 0.047$.

NonHDL-C concentration

The nonHDL-C concentration is defined as TPC concentration minus HDL-C concentration. A significant component was LDL, but also included IDL and VLDL (79, 90, 91). Within the fat-fed groups, the MONO group had a significantly lower nonHDL-C concentration than the SAT fat-fed groups, $P = 0.0093$ (Fig. 2A). High levels of dietary α-tocopherol reduced the plasma nonHDL-C concentration by 32% compared with fat-fed groups maintained on 25 IU α-tocopherol/kg-diet, $P = 0.021$ (Fig. 2B). Comparing the high and low α-tocopherol levels on the individual fat-fed groups showed that the nonHDL concentration of the POLY group on the 2,500 IU α-tocopherol/kg-diet supplement was 55% lower compared with the POLY group with 25 IU α-tocopherol/kg-diet, $P = 0.044$.
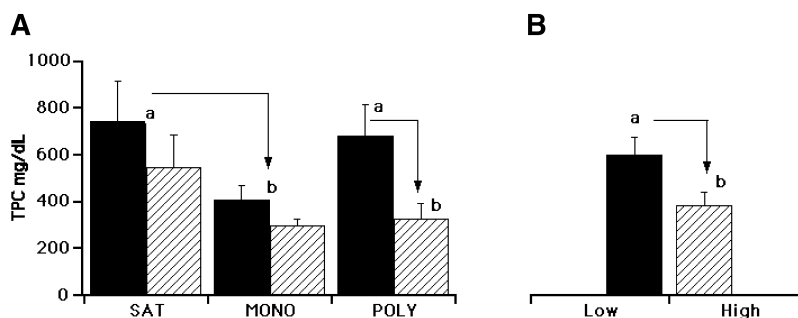

Fig. 1. α-Tocopherol lowers the plasma cholesterol concentration regardless of the dietary fat saturation. The results are given in mg/dl. The different fat-fed groups are shown for both low (filled) and high (hatched) levels of dietary α-tocopherol. Values are given as the mean ± SEM. A: Total plasma cholesterol (TPC) concentrations are shown for each diet group. The arrows and letters show a significant difference between diet group fed a diet enriched with saturated fats (SAT) and diet group fed a diet enriched with mono-unsaturated fats (MONO), $P = 0.007$. The TPC concentration was lower at high levels of dietary α-tocopherol compared with a diet with lower amounts of α-tocopherol across all fat groups. However, only the diet group fed a diet enriched with polyunsaturated fats (POLY) had a statistically significant reduction, $P = 0.041$. B: The TPC concentrations for low (filled) and high (hatched) levels of dietary α-tocopherol for all fat-fed groups were significantly different, $P = 0.017$.
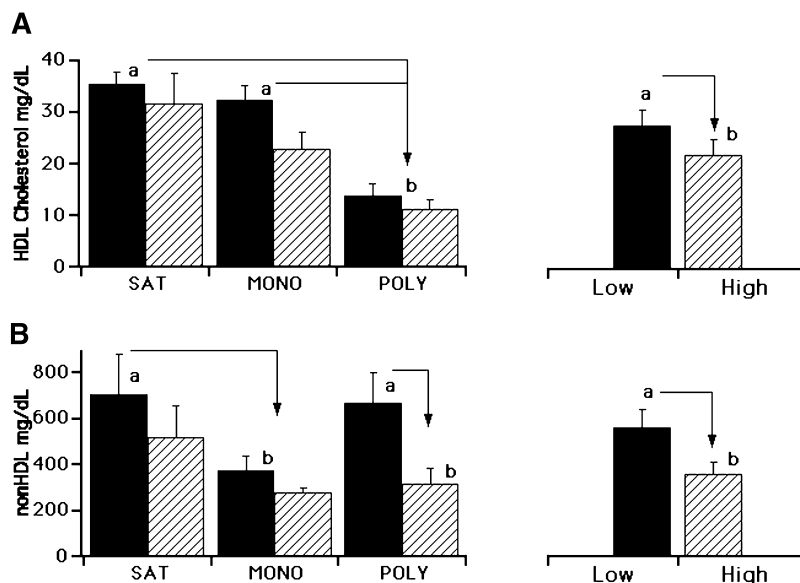
Fig. 2. A, left: HDL concentration was lower on the POLY diet compared with the SAT and MONO diets. Plasma HDL cholesterol (HDL-C) concentrations for both SAT and MONO were significantly higher than the POLY group, ANOVA $P < 0.0001$. A, right: Animals fed 2,500 IU α-tocopherol/kg-diet had lower HDL-C concentrations than those fed 25 IU α-tocopherol/kg-diet, ANOVA $P = 0.047$. Values are given as the mean ± SEM. B, left: The nonHDL-C concentration was lower on the MONO diet compared with the SAT and POLY diets with the smallest α-tocopherol supplement, ANOVA $P = 0.0093$. The POLY fat group fed 2,500 IU α-tocopherol/kg-diet had significantly lower nonHDL-C concentration than the POLY fat group fed 25 IU α-tocopherol/kg-diet, $P = 0.044$. B, right: Animals fed 2,500 IU α-tocopherol/kg-diet had lower non-HDL-C concentrations than those fed 25 IU α-tocopherol/kg-diet, ANOVA $P = 0.021$. Values are given as the mean ± SEM.

Plasma triglyceride concentration

There were no significant differences among the fat-fed groups, $P = 0.18$. Increasing the dietary α-tocopherol supplementation did not change plasma triglyceride levels, although there seemed to be a trend toward lower triglyceride concentrations at high levels of dietary α-tocopherol.

Plasma α-tocopherol

Overall plasma α-tocopherol was approximately 10-fold higher when the diet was supplemented with 2,500 IU α-tocopherol/kg-diet as compared with a diet supplemented with 25 IU α-tocopherol/kg-diet, $P < 0.0001$. The concentration of plasma α-tocopherol increased as TPC concentration increased for both high and low dietary α-tocopherol supplementation, $r = 0.76$ ($P = 0.0006$) and 0.85 ($P < 0.0001$), respectively (data not shown). The ratio of (μg plasma α-tocopherol)/(mg TPC) was not affected by the type of fat at either low (0.19 ± 0.05) or high dietary (3.3 ± 1.2) α-tocopherol, $P = 0.35$ and 0.88, respectively.

Artery cholesterol

The amount of cholesterol extracted from approximately 50 mg wet weight of the t1 segment of each of the diet group showed large variation about the mean. The results are summarized in **Fig. 3A**, **B**. Because variances about the means increased in proportion to the means, the results were analyzed after log transformation. Arter-

ies from SAT and POLY fat-fed diet groups contained about 3-fold more ester cholesterol than the arteries from CHOW-fed animals, $P < 0.0001$ and 0.0019, respectively (data not shown). Arteries from MONO fat-fed animals contained less ester cholesterol than did arteries from SAT-fed animals, $P = 0.006$. Figure 3C shows that log μg ester cholesterol from the arteries was linearly related to the TPC concentration, $r = 0.61$ ($P = 0.0002$).

Mean arterial ester cholesterol extracted from the t1 segment of the POLY fat-fed group on 2,500 IU α-tocopherol/kg-diet was 10-fold smaller than mean ester cholesterol from animals fed 25 IU α-tocopherol/kg-diet. The unpaired Student's t-test for high and low α-tocopherol in the POLY group gave $P = 0.088$. However, the Wilcoxon signed rank test suggested that there may be a significant difference in cholesterol levels between high and low dietary α-tocopherol, $P = 0.043$.

Artery α-tocopherol

At low levels of dietary α-tocopherol, 25 IU/kg-diet, there were no significant differences in the α-tocopherol levels among the fat-fed diet groups, $P = 0.33$. The arteries of both the MONO and POLY groups had significantly more artery α-tocopherol with a diet of 2,500 IU α-tocopherol/kg-diet compared with arteries from animals fed 25 IU α-tocopherol/kg-diet, $P = 0.037$ and 0.011 for MONO and POLY, respectively. At the high level of dietary α-tocopherol supplementation, arteries from the POLY fat-fed group contained more α-tocopherol than the
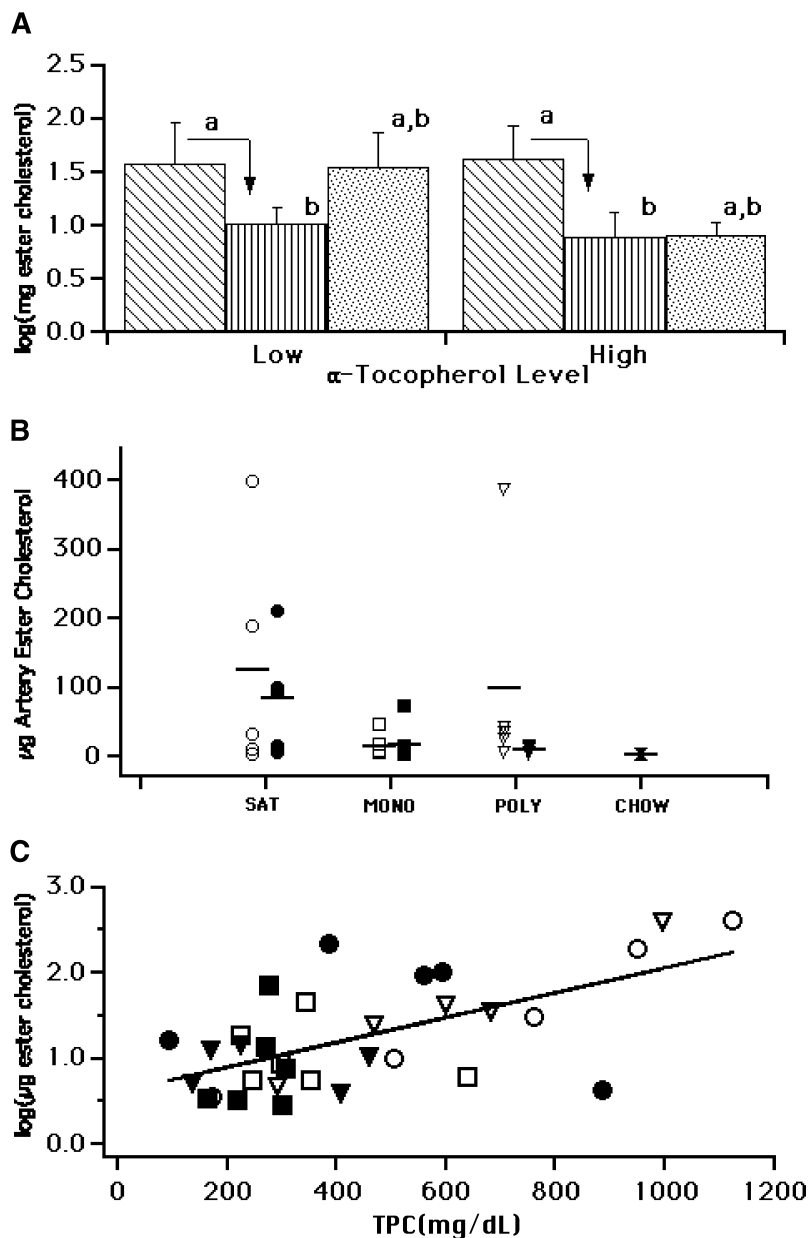
Fig. 3. Ester cholesterol accumulation in the t1 segment of rabbit arteries was highest in the SAT fat-fed group. A: A plot of the log (mg ester cholesterol) extracted from the t1 artery tissue segment: SAT (hatched), MONO (vertical), and POLY (dots). Values are given as the mean ± SEM. The statistically significant differences were found for SAT group compared with the MONO fat-fed group, $P = 0.006$. For the POLY fat-fed group the Wilcoxon signed rank test suggests that there may be statistically significant differences between high and low dietary α-tocopherol, $P = 0.043$. B: Distribution of arterial ester cholesterol for the different diet groups: filled 2,500 IU α-tocopherol/kg-diet; open 25 IU α-tocopherol/kg-diet; SAT, circle; MONO, square; and POLY, triangle. The mean value for the chow-fed animals is shown for comparison. C: A plot of the log (µg ester cholesterol per 50 mg t1 artery segment) versus TPC (mg/dl). The solid line is the linear fit of the data, $r = 0.61$ ($P = 0.0002$).

MONO and SAT fat-fed groups, $P = 0.014$ and 0.0091, respectively, as shown in **Fig. 4**.

There was no correlation between α-tocopherol and either total cholesterol or the ester cholesterol in the artery samples, $P > 0.89$ (data not shown). The (ng α-tocopherol)/(µg total cholesterol) ratio for artery tissues was approximately 1.2 for all groups at 25 IU α-tocopherol/kg-diet. The ratio was higher with 2,500 IU α-tocoph-

erol/kg-diet: 1.8 ± 1.6, 3.8 ± 1.7, and 10.9 ± 4.8 for SAT, MONO, and POLY arteries, respectively. For all the groups taken together, the ratio of (ng α-tocopherol)/(µg total arterial cholesterol) was larger with 2,500 IU α-tocopherol/kg-diet, $P = 0.0014$, compared with the 25 IU α-tocopherol/kg-diet. POLY-fed animals had the largest increase in the (ng α-tocopherol)/(µg total cholesterol ratio), $P = 0.0023$.

Fatty acid composition of lipids extracted from plasma and artery tissue

Dietary fat composition varied considerably among the three groups. The mole ratio of plasma saturated-monounsaturated-polyunsaturated fatty acids for SAT, MONO, and POLY were 32:49:18, 22:52:16, and 25:31:44, respectively. The fatty acid composition of the artery was rich in saturated fats compared with the plasma: 45:32:23, 44:36:20, and 40:22:37 for SAT, MONO, and POLY, respectively. Analysis confirmed that the arteries of the POLY group were higher in polyunsaturated fatty acids, $P < 0.0001$, and lower in monounsaturated fatty acids, $P = 0.0002$, compared with the other groups. The results are shown in **Fig. 5**. The mole ratios of saturated:monounsaturated:polyunsaturated fatty acids in the diets were 51:38:11, 14:67:18, and 9:13:77 for SAT, MONO, and POLY, respectively.

DISCUSSION

In this study, we sought to determine the effect of dietary fat and α-tocopherol on plasma lipoproteins and artery cholesterol. The principal findings of this study are as follows: first, regardless of fat type, rabbits supplemented with 2,500 IU α-tocopherol/kg-diet had TPC concentrations that were generally lower than rabbits supplemented with 25 IU α-tocopherol/kg-diet. The POLY fat group had the largest difference in TPC concentration between high and low α-tocopherol. Second, compared with diets supplemented with 25 IU α-tocopherol/kg-diet, diets with high dietary α-tocopherol had nonHDL plasma cholesterol concentrations were on the average 32% lower regardless of fat type while HDL concentrations were only slightly lower. Third, rabbits fed diets rich in MONO fat had significantly lower plasma TPC concentrations and less atherosclerosis that did rabbits fed SAT or POLY fats. And last, a dietary supplement of 2,500 IU α-tocopherol/

kg-diet for the POLY fat-fed group gave the same low level of atherosclerosis that was measured in the MONO fat-fed group.

With low levels of dietary α-tocopherol supplementation, the monounsaturated fat group had the lowest TPC concentration and the polyunsaturated fat group had a TPC concentration similar to that of the saturated fat group. In contrast, a previous study suggests that the TPC concentration in rabbits fed polyunsaturated fat would be lower than those fed monounsaturated fat if similar cholesterol supplements were used (90). Two other studies have reported that the TPC concentration of monounsaturated fat-fed rabbits was intermediate between saturated fat-fed and polyunsaturated fat-fed rabbits (92, 93). Studies with non-human primates reported that monounsaturated fats gave a TPC concentrations intermediate between saturated- and polyunsaturated-fat fed animals (12).

With high levels of dietary α-tocopherol, the TPC concentration of monounsaturated and polyunsaturated fat-fed animals were equivalent. Several studies of New Zealand white rabbits fed a cholesterol-rich diet supplemented with α-tocopherol have reported lower TPC concentrations compared with animals consuming low levels of dietary α-tocopherol (57–59, 61, 70, 71, 79), although two studies reported no change in TPC concentration (54, 74). In the absence of added cholesterol, α-tocopherol does not lower TPC (1, 75–77). In this study, the non-HDL fraction, e.g., VLDL, IDL, and LDL, underwent the greatest reduction with dietary α-tocopherol supplementation as has been reported in other studies (57, 58, 72). The studies reported herein demonstrated that the TPC concentration at high levels of α-tocopherol supplementation were lower for the polyunsaturated fats rich diet than for diets rich in either saturated and monounsaturated fats. Plasma HDL-C concentration was only slightly affected by α-tocopherol supplementation. The lower TPC concentration was mostly due to a reduction in the non-HDL-C concentration.

These studies show that the concentration of artery cholesteryl ester was directly proportional to the TPC concentration, suggesting that modification of plasma cholesterol was the primary factor in modulation of aortic cholesterol ester accumulation. Animals fed a monounsaturated diet had the lowest TPC and the least accumulation of cholesteryl ester in the artery. Other studies of cholesterol-induced atherosclerosis in rabbits have reported a correlation between the accumulation of arterial cholesterol and the TPC concentration. However, polyunsaturated fat-fed animals had less atherosclerosis in one study, and monounsaturated fat-fed animals less in the other (90, 92). The reason for these differences are not clear, but Kritchevsky has suggested that cholesterol-induced atherosclerosis in rabbits was sensitive to the ratio of dietary monounsaturated to saturated fats (29). What is notable in our studies is that compared with rabbits fed a diet containing low levels of α-tocopherol, a high level of dietary α-tocopherol was associated with a reduction in both TPC concentration and arterial cholesteryl ester. At
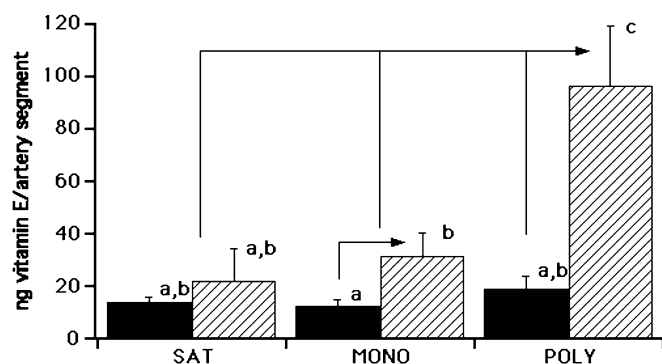

Fig. 4. A plot of (ng α-tocopherol)/t1artery segment for each of the fat-fed diet groups split by low (solid) and high (hatched) levels of α-tocopherol. High levels of dietary α-tocopherol significantly increased the amount of α-tocopherol in the artery lipids of the MONO and POLY fat groups, $P = 0.037$ and 0.011, respectively. The POLY fat-fed group had more α-tocopherol than either the MONO or SAT fat-fed groups, $P = 0.014$ and 0.0091 respectively.
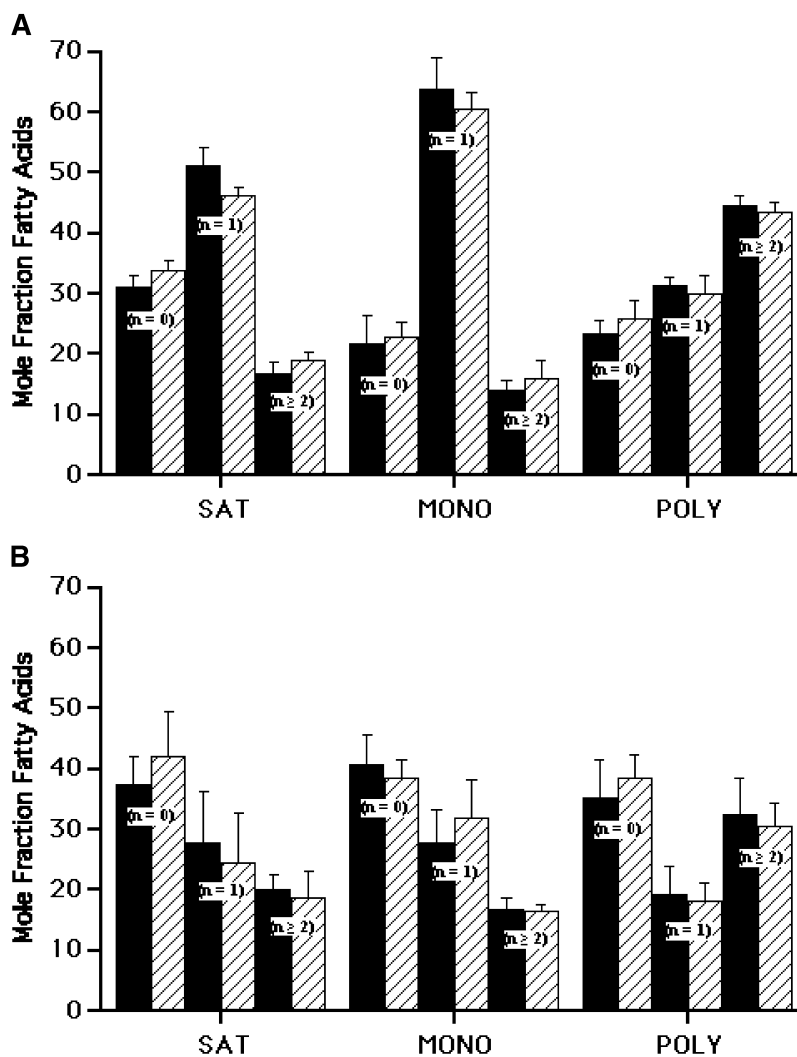
Fig. 5. A plot of the distribution of saturated (n = 0), monounsaturated (n = 1), and all polyunsaturated (n ≥ 2) fatty acids in the plasma lipids (A) and plaque lipids (B) of each fat-fed diet group. Solid bars represent groups with low levels of dietary α-tocopherol. Hatched bars are used for groups with high levels of dietary α-tocopherol. The plasma lipids in the different fat-fed groups reflect the diet composition. Saturated fatty acids, n = 0, are a more significant component of the plaque lipids. The fatty acid distribution in arteries from the SAT and MONO groups are similar to one another. The POLY group retains a significant fraction of the polyunsaturated fatty acids, n ≥ 2.

the higher concentrations of dietary α-tocopherol, the TPC and arterial cholesteryl ester of the POLY and MONO fat-fed groups were almost identical. These results suggest that α-tocopherol may play an important role in the trafficking of plasma lipids.

The concentration of plasma α-tocopherol was linearly related to the concentration of plasma cholesterol and the ratio of (μg plasma α-tocopherol)/mg TPC at low dietary (0.2) or high dietary (3.3) α-tocopherol, was the same irrespective of the type of fat. However, in the artery the ratio of α-tocopherol/total cholesterol at the high concentration of α-tocopherol depended on the predominant type of fat in the diet, suggesting a process that favors α-tocopherol accumulation or retention when the arterial fat contains more polyunsaturated fatty acid. Discrimination may be one of two types. The first is the loss of

α-tocopherol from the arteries by oxidation and catabolism. In vitro oxidation of LDL and HDL is more rapid in particles with greater concentrations of polyunsaturated fatty acids (8, 94). The greater sensitivity to oxidation would suggest that more α-tocopherol would be lost from lipids rich in polyunsaturated fatty acids, but exactly the opposite was true. The second type of discrimination may be related to a differential solubility of α-tocopherol in the fat deposited in artery tissue. In the plasma, the concentration of α-tocopherol was independent of fat type. Therefore, the high concentration of α-tocopherol in artery fats enriched in polyunsaturated fatty acids may suggest that the physical state of lipid deposits in the artery are different from the physical state of lipids in lipoprotein particles.

Previous studies in nonhuman primates have shown

that the amount of F$_2$-isoprostanes (lipid oxidation products produced by the free radical autoxidation of arachidonic acid) increased with the concentration of polyunsaturated fatty acids in arteries of SAT fat-fed animals (95). The increased levels of F$_2$-isoprostanes in arteries rich in saturated fats may be a consequence of lower α-tocopherol levels. Our studies suggest that the concentration of arterial α-tocopherol may be an important factor for assessing the protective role of α-tocopherol.

Studies using apoE deficient mice and New Zealand White rabbits reported that α-tocopherol reduces atherosclerosis (1, 79, 96–98). Other groups, however, report there was no reduction in atherosclerosis in mice supplemented with α-tocopherol (99–101). However, none of these studies examined the role of dietary fat. We find that high levels of dietary α-tocopherol lowered the TPC concentration and reduced atherosclerosis in rabbits fed cholesterol and fat, with the greatest effect when the dietary fat was polyunsaturated. We propose that high levels of dietary α-tocopherol may provide protection against atherosclerosis and suggest that the magnitude of α-tocopherol mediated protection may depend on the predominant type of fat in the diet.

These studies used α-tocopherol supplements that, for a 180 lb human would equal about 40 IU α-tocopherol/day for the low-level supplementation and about 4,000 IU α-tocopherol/day for the high supplementation. Our results suggest that higher levels of α-tocopherol may have beneficial health effects, but do not imply that 4,000 IU α-tocopherol/day would be an optimal intake for humans. The reduction of the TPC concentration with α-tocopherol supplementation was greater for the polyunsaturated fat diet. Therefore, our studies suggest that the intake of polyunsaturated fatty acids should be included when assessing the role of α-tocopherol.

The antioxidation properties of α-tocopherol have received much attention as a possible mechanism to reduce atherosclerosis. The reduction in atherosclerosis associated with the high levels of arterial α-tocopherol is consistent with the antioxidant hypothesis. However, several studies show that α-tocopherol may modulate certain enzyme activities like the cholesterol ester exchange protein (2, 63), PKC (61, 102, 103), and SR-B1 (72). Therefore, α-tocopherol affects biochemical pathways at the level of signaling and control in addition to its suggested role as an antioxidant (104, 105). When the physiological roles for α-tocopherol are better understood, it will be easier to design clinical trials and interpret the results from these trials.

In summary, these studies have demonstrated that rabbits fed a diet containing 0.1% (w/w) cholesterol, 25 energy % fat, and high levels of dietary α-tocopherol generally had lower TPC concentrations than rabbits fed low levels of α-tocopherol. High levels of α-tocopherol appeared to have the greatest effect when polyunsaturated fat was the predominant fat in the diet. Because the deposition of cholesteryl ester in arterial plaque paralleled the TPC concentration, our results suggest that high levels of dietary α-tocopherol may substantially reduce atherosclerosis by lowering TPC. The studies reported herein do not identify the respective contribution of antioxidation and enzyme regulation to the reduction in atherosclerosis. However, because both plasma and arterial cholesterol levels were lower with high levels of α-tocopherol supplementation, these studies suggest that α-tocopherol may have a significant role in modifying the activity of enzymes associated with remodelling lipids in the plasma compartment.🔳

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