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**Research Article**

Abnormalities in endothelium-dependent arterial relaxation develop early in atherosclerosis and may, in part, result from the effects of modified low-density lipoprotein (LDL) on agonist-mediated endothelium-derived relaxing factor (EDRF) release and EDRF degradation. alpha-Tocopherol (AT) is the main lipid-soluble antioxidant in human plasma and lipoproteins, therefore, we investigated the effects of AT on endothelium-dependent arterial relaxation in male New Zealand White rabbits fed diets containing (a) no additive (controls), (b) 1% cholesterol (cholesterol group), or 1% cholesterol with either (c) 1,000 IU/kg chow AT (low-dose AT group) or (d) 10,000 IU/kg chow AT (high-dose AT group). After 28 d, we assayed endothelial function and LDL susceptibility to ex vivo copper-mediated oxidation. Acetylcholine- and A23187-mediated endothelium-dependent relaxations were significantly impaired in the cholesterol group ($P < 0.001$ vs. control), but preserved in the low-dose AT group ($P$ = NS vs. control). Compared to the control and cholesterol groups, vessels from the high-dose AT group demonstrated profound impairment of arterial relaxation ($P < 0.05$) and significantly more intimal proliferation than other groups ($P < 0.05$). In normal vessels, alpha-tocopherol had no effect on endothelial function. LDL derived from both the high- and low-dose AT groups was more resistant to oxidation than LDL from control animals ($P < 0.05$). These data indicate that modest dietary treatment with AT preserves endothelial vasodilator function in cholesterol-fed rabbits […]



# Low-Dose α-Tocopherol Improves and High-Dose α-Tocopherol Worsens Endothelial Vasodilator Function in Cholesterol-Fed Rabbits

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## Abstract

**Abnormalities in endothelium-dependent arterial relaxation develop early in atherosclerosis and may, in part, result from the effects of modified low-density lipoprotein (LDL) on agonist-mediated endothelium-derived relaxing factor (EDRF) release and EDRF degradation. α-Tocopherol (AT) is the main lipid-soluble antioxidant in human plasma and lipoproteins, therefore, we investigated the effects of AT on endothelium-dependent arterial relaxation in male New Zealand White rabbits fed diets containing (*a*) no additive (controls), (*b*) 1% cholesterol (cholesterol group), or 1% cholesterol with either (*c*) 1,000 IU/kg chow AT (low-dose AT group) or (*d*) 10,000 IU/kg chow AT (high-dose AT group). After 28 d, we assayed endothelial function and LDL susceptibility to ex vivo copper-mediated oxidation. Acetylcholine- and A23187-mediated endothelium-dependent relaxations were significantly impaired in the cholesterol group ($P < 0.001$ vs. control), but preserved in the low-dose AT group ($P$ = NS vs. control). Compared to the control and cholesterol groups, vessels from the high-dose AT group demonstrated profound impairment of arterial relaxation ($P < 0.05$) and significantly more intimal proliferation than other groups ($P < 0.05$). In normal vessels, α-tocopherol had no effect on endothelial function. LDL derived from both the high- and low-dose AT groups was more resistant to oxidation than LDL from control animals ($P < 0.05$). These data indicate that modest dietary treatment with AT preserves endothelial vasodilator function in cholesterol-fed rabbits while a higher dose of AT is associated with endothelial dysfunction and enhanced intimal proliferation despite continued LDL resistance to ex vivo copper-mediated oxidation. (*J. Clin. Invest.* 1994. 93:844–851.) Key words: antioxidants • endothelium-derived relaxing factor • hypercholesterolemia • low density lipoproteins • vitamin E**

## Introduction

In 1980, Furchgott and Zawadzki (1) demonstrated the release of an endothelium-derived relaxing factor (EDRF)[1] in response to acetylcholine. Subsequent studies have shown that EDRF is important in the local control of vascular tone (2) and platelet aggregation (3). Abnormalities in endothelium-dependent arterial relaxation develop early in the course of atherogenesis (4) and may, in part, result from the effects of oxidized LDL (ox-LDL) on agonist-mediated EDRF release (5, 6) and EDRF degradation (7). Modified LDL inhibits receptor-mediated endothelium-dependent arterial relaxation (5, 6) and signal transduction (8) in vitro. Moreover, ox-LDL is cytotoxic to endothelial cells (9) and chemotactic for monocytes (10) leading to the accumulation of vascular inflammatory cells and the production of oxygen-derived free radicals, that can degrade EDRF (11, 12).

The most abundant lipid-soluble antioxidant in human plasma and LDL is α-tocopherol (13), and the oxidation of LDL in vitro is limited by α-tocopherol (14). Dietary supplementation with α-tocopherol results in enhanced LDL α-tocopherol content (15) and protection of LDL from copper- (15) and cell-mediated (14) oxidation in vitro. Plasma levels of α-tocopherol are inversely correlated with the development of angina pectoris (16, 17), and recent epidemiologic studies indicate that dietary vitamin E consumption is inversely associated with the development of coronary artery disease in both men (18) and women (19).

Despite evidence linking LDL oxidation to abnormal endothelium-dependent arterial relaxation and atherogenesis, the effect of antioxidants and, in particular, α-tocopherol on endothelial control of vascular tone remains poorly defined. Thus, we sought to examine the effect of α-tocopherol on the development of abnormal endothelium-dependent arterial relaxation in a cholesterol-fed rabbit model.

## Methods

*Materials.* Ketamine hydrochloride was purchased from Aveco Co., Inc., Fort Dodge, IA, and sodium pentobarbital was obtained from Anthony Products Co., Arcadia, CA. Sodium nitroprusside was obtained from Abbott Laboratories, North Chicago, IL. Vacutainer tubes were obtained from Beckton, Dickinson & Co., Rutherford, NJ, and glutaraldehyde, formaldehyde, osmium tetroxide, and cacodylate were purchased from Polysciences, Inc., Warrington, PA. Chelex 100 resin (100–200 mesh) was obtained from Bio-Rad Laboratories, Hercules, CA, and porcine intestinal mucosal heparin was purchased from Elkins-Sinn, Inc., Cherry Hill, NJ. α-Tocopherol acetate (95% pure), calcium ionophore (A23187), acetylcholine hydrochloride, norepinephrine, potassium bromide (KBr), cupric chloride ($CuCl_2$), Lowry protein assay kits (P5656), and all other compounds were purchased from Sigma Chemical Co., St. Louis, MO.

Krebs-Heinseleit-Indomethacin buffer (KHI) contained 118.3 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM $KH_2PO_4$, 25 mM $NaHCO_3$, 11.1 mM glucose, 10 μM indomethacin, and 0.026 mM $Na_2EDTA$. PBS consisted of 10 mM $NaP_i$, 0.15 M

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1. *Abbreviations used in this paper:* EDRF, endothelium-derived relaxing factor; ox-LDL, oxidized LDL; TC, total cholesterol; TG, triglycerides.

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NaCl, pH 7.4. Reagents used for LDL experiments were prepared with Chelex-treated, double-distilled, deionized water in order to prevent premature LDL oxidation catalyzed by trace amounts of transition metal ions. A23187 was prepared and diluted in 2% DMSO while all other reagents were prepared with distilled water.

*Animal subjects.* 48 male New Zealand White rabbits (2.4–3.6 kg) were exposed to dietary treatment for a period of 28 d. 12 of these animals were fed standard Purina rabbit laboratory diet (without vitamin mix; Rabbit Chow, Ralston Purina Co., St. Louis, MO) and served as the control group. 36 animals were fed a diet containing 1% (wt/wt) cholesterol with the following supplements ($n$ = 12 per group): (*a*) no additive (cholesterol group); (*b*) 1,000 IU/kg chow $\alpha$-tocopherol acetate (low-dose $\alpha$-tocopherol group); and (*c*) 10,000 IU/kg chow $\alpha$-tocopherol acetate (high-dose $\alpha$-tocopherol group). An additional four age-matched rabbits were used to evaluate the effect of $\alpha$-tocopherol on endothelium-dependent arterial relaxation in the absence of hypercholesterolemia. These doses of $\alpha$-tocopherol were chosen in order to provide rabbits with 25- and 250-fold the nutritional requirements for vitamin E. All diets were prepared by a commercial vendor (Research Diets Inc., New Brunswick, NJ) and animals consumed 110 g/d of chow and allowed water ad libitum. Blood was obtained in Vacutainer tubes (4.5 mg $Na_2EDTA$/3 ml) before dietary treatment and at the time of sacrifice. Plasma was prepared by centrifugation (1,000 *g*) for 11 min at 4°C and stored at −70°C (protected from light) for subsequent assay of plasma lipoproteins and $\alpha$-tocopherol.

Plasma total cholesterol (TC) (20) and triglycerides (TG) (21) were quantified in using enzymatic methods. HDL-cholesterol (HDL-C) was measured following phosphotungstic-$MgCl_2$ precipitation of apoliprotein B containing VLDL and LDL (22). Plasma LDL + VLDL cholesterol (LDL-C + VLDL-C) was derived from the above data using the modified Friedewald formula: LDL-C + VLDL-C = TC − (HDL-C + 0.16 × TG). In hypercholesterolemic animals, assays were performed on plasma diluted with PBS.

*In vitro assay of vascular function.* Rabbits were killed with pentobarbital (120 mg/kg) via a marginal ear vein and the thoracic aorta excised. The aorta was immediately placed in ice-cold KHI and extraneous tissue carefully removed. The aorta was cut into 3-mm rings (six to eight rings per animal), placed in an organ chamber (Radnoti Glass Co., Monrovia, CA) containing 20 ml KHI (37°C, pH 7.4), aerated with 95% $O_2$/5% $CO_2$, and suspended between two tungsten stirrups. One stirrup was connected to a force transducer (model FT-03, Grass Instrument Co., Quincy, MA) for the measurement of isometric tension, which was recorded on a chart recorder (model RS 3800, Gould Instrument Co., Columbus, OH). Each vessel ring was prestretched with 2.5 g tension and allowed to equilibrate for 90 min before the introduction of vasoactive drugs. In some experiments, the vascular endothelium was removed by gentle rubbing with a moistened cotton swab. At the end of selected experiments, a representative ring was fixed in 10% formalin and subjected to scanning electron microscopy to confirm the presence or absence of endothelium.

Endothelial control of vascular tone was assayed by the addition of the muscarinic agonist acetylcholine (final concn. $10^{-9}$ to $10^{-5}$ M) and the calcium ionophore A23187 ($10^{-9.5}$ to $10^{-6}$ M), while smooth muscle cell vasodilator function was assayed using sodium nitroprusside ($10^{-9}$ to $10^{-5}$ M). Smooth muscle cell contractile function was assayed by the addition of norepinephrine ($10^{-9}$ to $10^{-5}$ M).

*Plasma and aortic antioxidant content.* Plasma (0.5 ml) was precipitated with an equal volume of ethanol, extracted twice with equal volumes of hexane, and the combined hexane phases dried under nitrogen in an amber glass vial. Dried samples were resuspended in 0.5 ml of ethanol for analysis. Aortic samples were snap frozen in liquid nitrogen at the time of sacrifice and stored (protected from light) at −70°C until analysis. Aortic samples (0.1–0.35 g) were cleaned of extraneous tissue and homogenized in ice-cold potassium chloride. Homogenates were incubated at 70°C with 25% sodium ascorbate for 5 min and saponified for 30 min with 10 N potassium hydroxide. Samples were extracted twice with hexane at room temperature and washed with 1 ml of distilled water, and the hexane was phase dried under nitrogen. The residue was resuspended in 1 ml ethanol and centrifuged at 1,000 *g*, and the pellet was extracted with 1 ml of ethanol. The collected ethanol was dried under nitrogen and resuspended in 25 μl of methylene chloride and 75 μl of ethanol for analysis. Sample extracts were analyzed by using UV detection (model 167, Beckman Instruments, Inc., Palo Alto, CA) at 286 nm after reverse-phase HPLC with a C18 Baker Bond (5 μm) column and methanol/tetrahydrofuran/water/methylene chloride (70/18/7/5) containing 1% ammonium acetate as the mobile phase. This system was calibrated daily using D,L-$\alpha$-tocopherol in ethanol.

*LDL oxidation studies.* The isolation and oxidation of LDL were performed as described by Retsky and colleagues (23). Briefly, blood (10 ml) was collected from fasting rabbits into sodium heparin Vacutainer tubes (sodium heparin 286 USP U/15 ml blood) and plasma was obtained as described above. Residual aqueous antioxidants such as ascorbate and urate were removed from plasma by gel filtration using Sephadex G-25-300 (Pharmacia, Uppsala, Sweden) (23). LDL was prepared from fresh-filtered plasma by the method of Chung and colleagues (24) using single vertical spin discontinuous density gradient ultracentrifugation. Plasma was adjusted to a density of 1.21 g/ml by the addition of solid KBr, and LDL was isolated by centrifugation (443,000 $g_{av}$) at 80,000 rpm for 45 min using a Beckman NVT90 rotor and a Beckman L8-80M ultracentrifuge. Chelex was added to the collected LDL fraction to bind metal ions, and the resin removed using a 0.2-μm syringe filter (Gelman Sciences Inc., Ann Arbor, MI). The isolated LDL was used immediately for experiments. Protein content was determined by the method of Lowry et al. (25) using BSA as a standard.

Standard incubation of LDL for susceptibility to oxidation was performed at a concentration of 0.1 mg of LDL protein/ml in the presence of 1.25 μM $CuCl_2$ at 37°C. Lipid peroxidation was determined by assay of sample absorbance at 234 nm (diene conjugation) in 10 minute intervals using an Hitachi U-2000 spectrophotometer equipped with a thermostatic six-cell holder and a magnetic stirring device. LDL susceptibility to lipid peroxidation was quantified by the lag phase duration before the propagation phase of diene conjugation as described by Esterbauer and colleagues (26).

*LDL antioxidant content.* A 200-μl sample containing 0.1 mg of LDL protein in PBS was extracted with 200 μl of methanol and 2.5 ml of hexane. The hexane phase (2.0 ml) was dried under nitrogen, resuspended in ethanol, and analyzed by reverse-phase HPLC using an LC-8 column (Supelco, Inc., Bellfonte, PA) and 1% water in methanol containing 10 mM lithium perchlorate as mobile phase (27). The eluate was analyzed by electrochemical detection at an applied potential of 0.6 V in an LC 4B amperometric electrochemical detector (Bioanalytical Systems, West Lafayette, IN). Calibration of the HPLC system was performed daily using fresh solutions of D,L-$\alpha$-tocopherol in ethanol.

*Pathologic examination.* Four to six animals from each group were used for pathologic examination. The thoracic aorta was isolated as described above and cannulated with a 16-gauge stainless steel cannula. A 1–2-cm portion of the aortic arch was snap frozen in liquid nitrogen and stored at −70°C (protected from light) for determination of tissue $\alpha$-tocopherol levels (see above). The remaining thoracic aorta was cleared of blood by infusion of PBS for 5 min followed by perfusion under physiologic pressure (90 mmHg) with a solution of 10% formalin in PBS (pH 7.4) for 20 min. The thoracic aorta was gently cleaned of loose connective tissue, washed in cacodylate-sucrose buffer (10.26 g sucrose in 150 ml 0.1 M cacodylate) for 5 min, and fixed in glutaraldehyde-cacodylate solution (3% glutaraldehyde, 0.1 M cacodylate) for 24 h. Samples prepared in this manner were stored at 4°C in cacodylate-sucrose buffer for histologic examination. Histologic examination was performed on 2-mm arterial segments obtained from proximal, mid, and distal portions of the aortic arch, which were dehydrated and embedded in paraffin by standard histologic procedures. Sections were stained with resorcin fuchsin (for elastin) and subjected to morphometric analysis of intimal and medial area using an automated videomicroscopy system (Image Technology Corp., Deer Park, NY). Fixed tissues were prepared for scanning electron microscopy by postfixation in 1%

osmium tetroxide and dehydration using graded ethanol exposure. Sections were dried in hexamethyldisilazane, coated with gold and palladium, and observed in an AMR 1,000Å scanning electron microscope (AMRAY, Bedford, MA).

*Data analysis.* Unless otherwise specified, all values are presented as a mean±SD. The vascular responses to the agents acetylcholine, A23187, and sodium nitroprusside are reported as the percent relaxation compared to the contraction produced by 1 μM norepinephrine. Contractile responses to norepinephrine are expressed as the grams of force generated to a 1 μM dose. The dose responses to acetylcholine, A23187, and sodium nitroprusside were compared within groups using ANOVA with a repeated measures design. The vascular responses due to dietary therapy were compared with two-way ANOVA. Comparisons among groups for lipoprotein levels, vascular contraction, antioxidant levels, and intimal-to-medial ratio were performed using ANOVA with a post hoc Neuman-Keuls comparison. Statistical significance was accepted if the null hypothesis was rejected at the $P < 0.05$ level.

## Results

*Plasma cholesterol and antioxidant levels.* The plasma cholesterol and α-tocopherol levels in all four dietary treatment groups are contained in Table I. The baseline characteristics of all animals were similar prior to dietary treatment. Animals fed standard laboratory diet (control group) demonstrated plasma total cholesterol, LDL + VLDL cholesterol, HDL cholesterol, and triglyceride levels of 70±25, 21±14, 29±11, and 122±43 mg/dl, respectively. Animals receiving diets containing 1% cholesterol for 28 d demonstrated significant elevations of plasma total cholesterol, LDL + VLDL cholesterol, HDL cholesterol, and triglycerides compared to animals not receiving cholesterol ($P < 0.05$). Animals receiving 1% cholesterol diets supplemented with either low-dose or high-dose α-tocopherol demonstrated plasma lipoprotein levels that were not significantly different from the cholesterol group (Table I).

The plasma α-tocopherol levels were consistent with the dietary content of α-tocopherol. Control animals demonstrated a plasma α-tocopherol level of 6.2±1.0 μM. The cholesterol diet alone was associated with a 2.5-fold elevation in plasma α-tocopherol content to 16.6±4.6 μM ($P < 0.05$ vs. control) which is likely a consequence of increased α-tocopherol absorption with a high fat diet and increased plasma capacity for lipid-soluble agents (28). In contrast, rabbits fed chow containing 1,000 and 10,000 IU/kg α-tocopherol demonstrated plasma α-tocopherol levels of 108.2±19.6 and 326.9±160.2 μM, respectively, both significantly elevated with respect to the control and cholesterol groups ($P < 0.05$). Similarly, aortic levels of α-tocopherol increased 10-fold in the low-dose α-tocopherol group ($P < 0.01$) and 80-fold in the high-dose α-tocopherol group ($P < 0.01$) compared to control animals. Aortic levels of α-tocopherol in the cholesterol group were elevated compared to controls (16.2±8.2 vs. 6.3±1.9 nmol/g) but this difference was not statistically significance ($P = 0.10$).

*α-Tocopherol and vascular reactivity.* The contractile response of aortic vessels to norepinephrine was similar among the dietary treatment groups. Vessels from animals in the control, cholesterol, low-dose α-tocopherol, and high-dose α-tocopherol groups demonstrated contractions to 1 μM norepinephrine of 3.0±0.8, 3.2±0.8, 3.4±1.0, and 3.7±0.9 g, respectively.

The effects of dietary cholesterol and α-tocopherol supplementation on acetylcholine-induced vasodilation is shown in Fig. 1. In the absence of endothelium, the response to acetylcholine was not significantly different among dietary groups (Fig. 1 *A*). In the presence of endothelium, vessels from rabbits fed standard rabbit lab diet demonstrated dose-dependent relaxation in response to acetylcholine with a maximal relaxation of 80±9% ($P < 0.001$). Rabbits from the cholesterol group demonstrated significantly impaired responses to acetylcholine with a maximal relaxation of 39±13% ($P < 0.001$ vs. control). In contrast, rabbits fed a 1% cholesterol diet supplemented with low-dose α-tocopherol demonstrated maximal acetylcholine-induced vasorelaxation (83±12%) that was not significantly different from control rabbits. Relaxations in vessels obtained from the high-dose α-tocopherol group, however, were abolished in response to acetylcholine (−11±15%), which was significantly different from both the control ($P < 0.001$) and cholesterol ($P < 0.05$) groups.

Dietary supplementation with α-tocopherol produced similar effects on endothelium-dependent vasorelaxation with the receptor-independent calcium ionophore A23187 (Fig. 2). In the absence of endothelium, the response to A23187 did not differ among dietary groups (Fig. 2 *A*). In the presence of endothelium, vessels harvested from control rabbits demonstrated significant dose-dependent relaxation in response to A23187 with a maximal response of 95%±7% ($P < 0.001$). The relaxation in vessels from the cholesterol group was significantly impaired relative to control animals with a maximal relaxation

*Table I. Plasma Lipoprotein Levels, Plasma α-Tocopherol Levels, and Aortic α-Tocopherol Content in Study Animals*

| | Control | Cholesterol | Low-dose α-tocopherol | High-dose α-tocopherol |
|---|---|---|---|---|
| Plasma lipoproteins | | | | |
| TC (*mg/dl*) | 70±25 | 1,834±555* | 1,510±302* | 1,785±575* |
| VLDL + LDL-C (*mg/dl*) | 21±14 | 1,494±603* | 1,135±434* | 1,314±630* |
| HDL-C (*mg/dl*) | 29±11 | 305±181* | 347±246* | 559±464* |
| TG (*mg/dl*) | 122±43 | 214±63* | 173±55 | 308±316* |
| α-Tocopherol levels | | | | |
| Plasma (*μM*) | 6.2±1.0 | 16.6±4.6* | 108.2±19.6* | 326.9±160.2*‡ |
| Aorta (*nmol/g*) | 6.3±1.9 | 16.2±8.2 | 63.2±22.5* | 506.3±258.2*‡ |

All values represent mean±SD of samples from 6–10 animals. * $P < 0.05$ compared to control group. ‡ $P < 0.05$ vs. cholesterol and low-dose α-tocopherol groups.

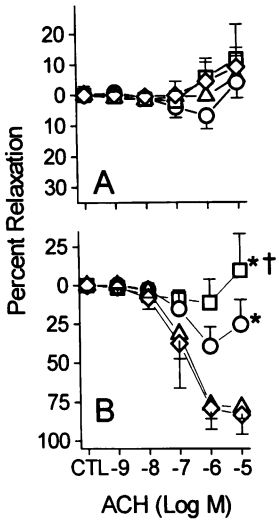


*Figure 1.* The effects of α-tocopherol on acetylcholine-induced endothelium-dependent vasorelaxation in cholesterol-fed rabbits. Aortic vessels were harvested from rabbits fed standard laboratory diet (△), 1% cholesterol diet (○), and 1% cholesterol diet with 1,000 IU/kg (◇) or 10,000 IU/kg (□) α-tocopherol and exposed to increasing concentrations of acetylcholine (*ACH*) in the absence (*A*) or presence (*B*) of endothelium. Vessels were precontracted with 1 μM norepinephrine and relaxation reported as the percent reduction in tension from that produced by norepinephrine. Values represent the mean±SD of vessels from 6–10 animals; $^{*}P < 0.001$ vs. control, $^{\dagger}P < 0.05$ vs. cholesterol group.

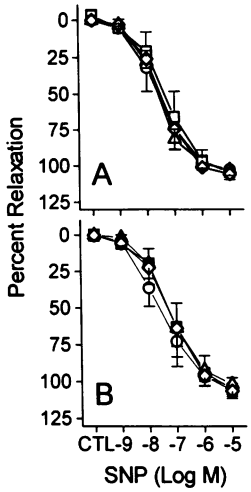


*Figure 3.* The effects of α-tocopherol on sodium nitroprusside-mediated vasorelaxation in cholesterol-fed rabbits. Vessels were harvested from rabbits fed standard laboratory diet (△), 1% cholesterol diet (○), and 1% cholesterol diet with 1,000 IU/kg (◇) or 10,000 IU/kg (□) α-tocopherol and exposed to increasing concentrations of sodium nitroprusside in the absence (*A*) or presence (*B*) of endothelium. Vessels were precontracted with norepinephrine (1 μM) and relaxation recorded as the percent reduction in force from that produced by norepinephrine. Values represent the mean±SD of vessels harvested from 6–10 animals.

of 59±7% ($P < 0.001$). This impairment in A23187-induced endothelium-dependent vasodilation was significantly improved in cholesterol-fed animals treated with low-dose α-tocopherol. Vessels harvested from these animals demonstrated dose-dependent relaxation similar to that of the control group ($P$ = NS) with a maximal relaxation of 87±11%. In contrast, vessels from the high-dose α-tocopherol group relaxed poorly to A23187 demonstrating a maximal response of 50±20%, which was significantly impaired relative to controls ($P < 0.001$) but similar to vessels from the cholesterol group ($P$ = NS).

Smooth muscle cell function was comparable among the dietary treatment groups (Fig. 3). Vessels from all four groups demonstrated dose-dependent vasodilation in response to increasing concentrations of sodium nitroprusside ($P < 0.001$) with no significant differences noted on the basis of dietary treatment or the presence of endothelium.

In order to assess the direct effects of α-tocopherol on endothelium-dependent arterial relaxation in the absence of hypercholesterolemia, we incubated vessels from normal rabbits with varying concentrations of α-tocopherol. Vessels derived from normal age-matched rabbits were incubated with 0, 100, 300, and 1,000 μM α-tocopherol acetate for 6 h and endothelium-dependent relaxation in response to acetylcholine examined. As shown in Fig. 4, there was no significant loss of endothelium-dependent relaxation in response to acetylcholine. This finding is particularly noteworthy since the maximal concentration of α-tocopherol in these incubations was three-fold greater than plasma α-tocopherol levels in the high-dose α-tocopherol group (1,000 vs. 326 μM, respectively).

*Pathologic examination of vascular tissue.* We used light microscopy as a means to evaluate the extent of intimal proliferation in the treatment groups used in this study. Microscopic analysis revealed similar intimal accumulation of lipids and foam cells among animals in the cholesterol and low-dose α-tocopherol groups. Vascular tissue derived from animals in the high-dose α-tocopherol group, however, demonstrated qualitatively more intimal thickening and intimal lipid accumulation than either the cholesterol or low-dose α-tocopherol groups. Using the ratio of intimal area to medial area as an index of intimal proliferation, aortae derived from the high-dose α-tocopherol group demonstrated an intimal-to-medial ratio of 0.359±0.041 (Fig. 5). This degree of intimal proliferation was significantly greater than that found in either the cholesterol group (0.138±0.027; $P < 0.05$) or the low-dose α-tocopherol group (0.174±0.044; $P < 0.05$). The intimal-to-medial ratio in the cholesterol group and low-dose α-tocopherol group were not significantly different ($P > 0.05$).

Representative scanning electron micrographs taken from aortae in this study are shown in Fig. 6. Control animals demonstrated normal endothelial cell morphology with alignment

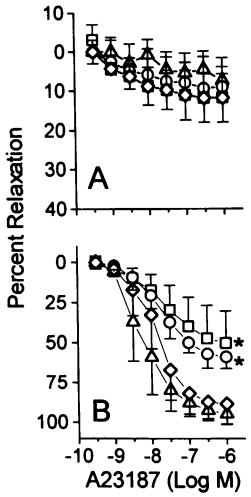


*Figure 2.* The effects of α-tocopherol on A23187-induced endothelium-dependent vasorelaxation in cholesterol-fed rabbits. Aortic vessels were harvested from rabbits fed standard laboratory diet (△), 1% cholesterol diet (○), and 1% cholesterol diet with 1,000 IU/kg (◇) or 10,000 IU/kg (□) α-tocopherol. Vessels were contracted with 1 μM norepinephrine and exposed to increasing concentrations of A23187 in the absence (*A*) or presence (*B*) of endothelium. Relaxation represents the percent reduction in force from that produced by norepinephrine. Values represent the mean±SD of vessels derived from six to ten animals; $^{*}P < 0.001$ vs. control.

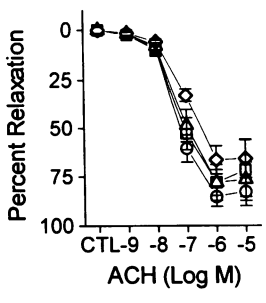


*Figure 4.* The effect of α-tocopherol on acetylcholine-mediated endothelium-dependent arterial relaxation in normal vessels. The thoracic aorta was harvested from normal rabbits, suspended in organ chambers, and incubated with KHI (□) or KHI containing 100 (○), 300 (△), or 1,000 (◇) μM α-tocopherol acetate. After 6 h, vessels were contracted with norepinephrine (1 μM) and cumulatively exposed to the indicated doses of acetylcholine (*ACH*). Results represent mean±SD of vessels harvested from four animals.

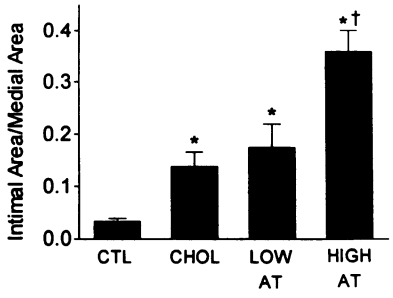


*Figure 5.* Dietary α-tocopherol and the extent of intimal proliferation. Thoracic aortae were harvested from rabbits fed standard laboratory diet (*CTL*), 1% cholesterol (*CHOL*), 1% cholesterol and low-dose α-tocopherol (*LOW AT*), or 1% cholesterol and high-dose α-tocopherol (*HIGH AT*). The vessels were fixed and prepared as described in Methods, embedded in paraffin, and stained with resorcin fuchsin (for elastin). Stained (0.2 mm) sections of aortic arch were subjected to morphometric analysis of intimal area and medial area using an automated videomicroscopy system. Values are displayed as mean±SD and represent analysis of nine sections per animal taken from three animals in each group. $*P < 0.05$ vs. control group, $^{\dagger}P < 0.05$ vs. cholesterol and low-dose α-tocopherol groups.

in the direction of flow and no intercellular gaps. Animals from the cholesterol group, in contrast, demonstrated endothelial cells without alignment in the direction of flow as well as raised areas of endothelial cells consistent with underlying foam cell formation. Similar findings were noted in the high- and low-dose α-tocopherol groups without any striking qualitative differences among any cholesterol-fed groups.

*LDL oxidation studies.* In order to determine whether changes in endothelial function with dietary antioxidant therapy may be a consequence of altered LDL resistance to oxidation, we examined the effect of dietary antioxidant treatment on the susceptibility of LDL to oxidation by copper ions. Exposure of LDL to copper ions results in the consumption of intraparticle antioxidants (tocopherols, ubiquinols, and carotenoids) followed by rapid formation of lipid hydroperoxides and conjugated dienes (26, 27). The lag phase duration before the propagation phase of diene conjugation can be used as an indicator of LDL resistance to oxidative modification (26). Therefore, we assayed plasma-derived LDL in the four treatment groups for α-tocopherol content and the formation of conjugated dienes in the presence of copper ions (Table II). LDL isolated from rabbits fed lab diet or 1% cholesterol alone demonstrated similar lag phase times of 135±42 and 160±66 min, respectively. In contrast, LDL isolated from animals fed cholesterol diets containing low- and high-dose α-tocopherol were more resistant to copper-mediated oxidation as evidenced by prolonged lag phase times of 183±18 and 219±53 min, respectively (both $P < 0.05$ vs. control). The LDL antioxidant content paralleled the dietary treatment (Table II). LDL derived from the control and cholesterol groups demonstrated similar α-tocopherol contents of 2.8±1.2 and 2.7±1.7 nmol/mg, respectively. Rabbits treated with low- and high-dose α-tocopherol demonstrated LDL α-tocopherol levels that were 7- and 20-fold elevated compared to controls (19.7±5.6 and 54.9±24.6 nmol/mg, respectively; $P < 0.05$ vs. control).

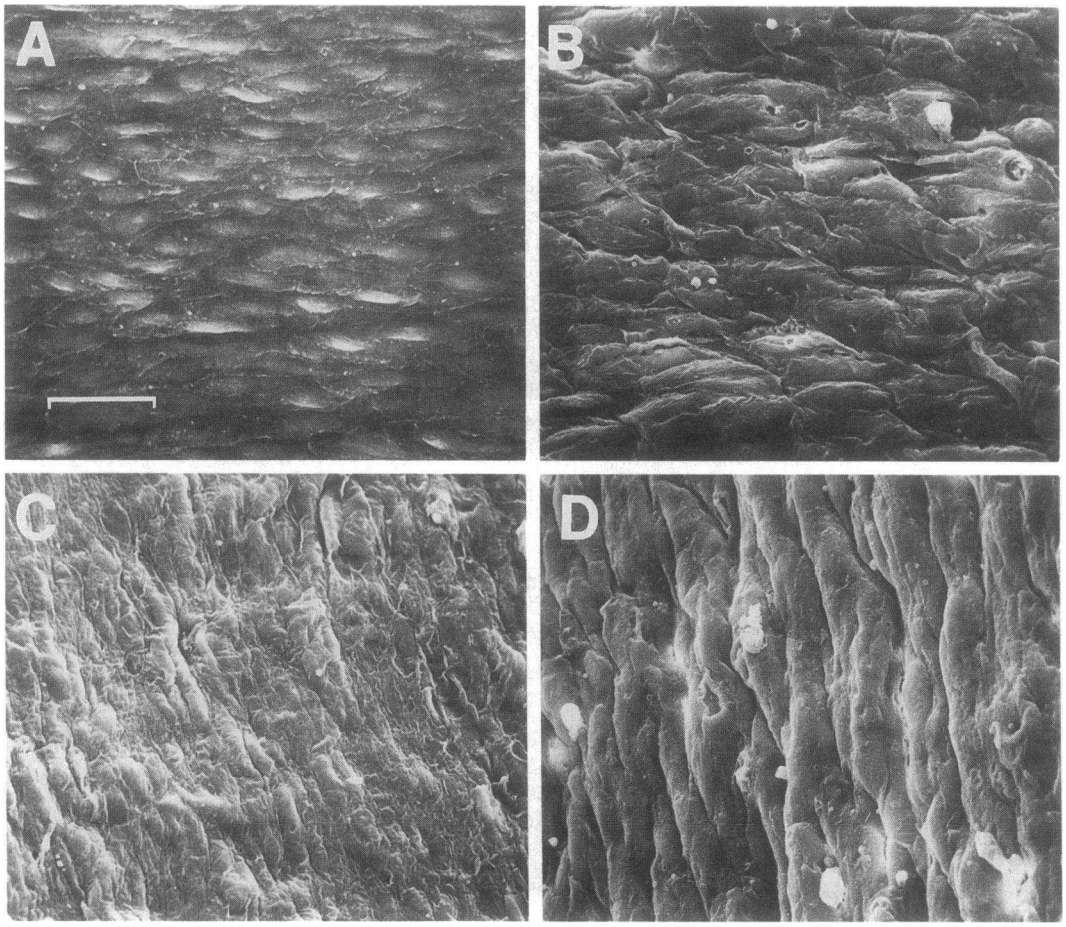


*Figure 6.* Representative scanning electron micrographs of aortic endothelium from animals fed standard laboratory diet (*A*), 1% cholesterol (*B*), 1% cholesterol and low-dose α-tocopherol (*C*), or 1% cholesterol and high-dose α-tocopherol (*D*). Animals fed standard diet alone (*A*) demonstrated normal endothelial cell morphology with cell alignment in the direction of flow and no intercellular gaps. In contrast, vessel endothelium from animals fed diets containing 1% cholesterol (*B–D*) demonstrated a loss of flow-oriented alignment, raised areas indicative of (presumptive) subendothelial foam cell formation, and occasional intercellular gaps. Qualitatively, endothelial morphology was similar in all the cholesterol-fed groups. The solid bar indicates the scale reference of 40 μm.

*Table II. LDL Susceptibility to Ex Vivo Copper-mediated Oxidation and LDL α-Tocopherol Levels*

| | Control | Cholesterol | Low-dose α-tocopherol | High-dose α-tocopherol |
|---|---|---|---|---|
| Lag phase (*min*) | 135±42 | 160±66 | 183±18* | 219±58* |
| LDL α-tocopherol (*nmol/mg LDL protein*) | 2.8±1.2 | 2.7±1.7 | 19.7±5.6* | 54.8±24.6*‡ |

All values represent mean±SD of samples from six animals. * $P < 0.05$ compared to control group. ‡ $P < 0.05$ vs. low-dose α-tocopherol group.

## Discussion

The data presented here demonstrate the preservation of endothelium-dependent vasodilator function in cholesterol-fed rabbits with a 25-fold nutritional excess of α-tocopherol (110 IU/day) while a 250-fold nutritional excess of α-tocopherol (1,100 IU/d) led to impaired endothelium-dependent arterial relaxation and enhanced intimal proliferation. In fact, vessels from the high-dose α-tocopherol group were less responsive to acetylcholine than animals fed cholesterol alone. This apparently harmful effect of high-dose α-tocopherol in cholesterol-fed rabbits was associated with a greater extent of intimal proliferation compared to animals in the low-dose α-tocopherol and cholesterol groups despite continued protection of LDL against copper-mediated oxidation. Impaired endothelial function in animals receiving the higher dose of α-tocopherol was not attributable to plasma lipoprotein levels or smooth muscle cell function.

With regard to the responses observed in the low-dose α-tocopherol group, the antioxidant properties of α-tocopherol could favorably affect endothelial vasodilator function. Normal arteries exposed to ox-LDL develop impaired endothelium-dependent arterial relaxation (5). Abnormalities in EDRF action caused by ox-LDL are related to lipid peroxidation products that accumulate during the oxidation process (29). Oxidative modification of LDL is associated with the conversion of lecithin to lysolecithin in the LDL particle, presumably through a phospholipase $A_2$ (30) or platelet-activating factor–acetylhydrolase (31) activity intrinsic to apolipoprotein B-100. Incubation of ox-LDL with defatted albumin (5) or phospholipase B (29) depletes the particle of lysolecithin and attenuates the development of abnormal vasomotion. In fact, direct incubation of normal rabbit aortae with lysolecithin alone results in inhibition of EDRF-mediated vessel relaxation (32). α-Tocopherol is a lipid-soluble antioxidant that inhibits cell-mediated oxidation of LDL (14). Thus, one possible mechanism for the beneficial effect of α-tocopherol on endothelial function in cholesterol-fed rabbits is the reduced formation of lysolecithin in the vascular wall by virtue of α-tocopherol-mediated increased resistance of LDL to oxidation in vivo.

Increased LDL resistance to oxidation may have other important implications. Clinically important LDL oxidation seems to occur primarily in vascular tissues (33) and, once modified, LDL is toxic to endothelial cells (9) and promotes the recruitment and retention of inflammatory cells into the vascular wall (10), presumably by inducing the production of monocyte chemotactic protein-1 (MCP-1) in the vascular wall (34). Monocytes and macrophages are capable of releasing oxygen-derived free radicals (35), possibly leading to continued LDL oxidation in the arterial wall and continued monocyte recruitment (10). The prevention of LDL oxidation by α-tocopherol limits the production of MCP-1 (34) and thus, should inhibit the recruitment of monocytes/macrophages into the vascular wall and prevent premature degradation of EDRF (7).

Investigation into the chemical nature of EDRF suggests that EDRF is either nitric oxide (36) or a related redox form thereof (37, 38) that combines readily with a number of chemical species including oxygen (39) and superoxide anion (40) leading to a loss of biologic activity (11). One must consider that the preservation of EDRF action in the low-dose α-tocopherol animals here may be a consequence of the free radical scavenging characteristics of α-tocopherol vis-à-vis superoxide anion. Alternatively, α-tocopherol may in some way inhibit superoxide production by endothelial cells. In fact, hypercholesterolemic vessels appear to produce excess superoxide anion (41) and enhanced degradation of superoxide anion in atherosclerotic rabbits improves the response to endothelium-dependent vasodilators (42).

Contemporary views of LDL oxidation and atherogenesis would imply that intimal proliferation should also be limited in the cholesterol-fed animals treated with low-dose α-tocopherol. In the present study, intimal proliferation in cholesterol-fed animals was not altered by treatment with low-dose α-tocopherol. Previous animal studies examining the effects of α-tocopherol on atherosclerosis have produced conflicting results. Studies performed in rabbits (43–45) using mildly atherogenic conditions (plasma total cholesterol of ≈ 250–400 mg/dl) have demonstrated diminished aortic lesions in animals consuming diets containing 0.5–1% (wt/wt) vitamin E. In contrast, rabbit studies employing atherogenic conditions similar to the present study (plasma cholesterol 1,000–2,000 mg/dl) have failed to demonstrate any benefit of dietary supplementation with vitamin E (28, 46). One must consider, therefore, that in the setting of severe hypercholesterolemia (as in the present study) the development of intimal proliferation may be less dependent upon the formation of ox-LDL in vivo and, therefore, limitation of LDL oxidation would not inhibit intimal proliferation in this model.

In contrast to the results with low-dose α-tocopherol, animals receiving 10-fold more α-tocopherol (1,100 IU/day) demonstrated a profound impairment of endothelium-dependent arterial relaxation. This impairment was associated with an increase in the extent of intimal proliferation compared to the other treatment groups. Most importantly, in these animals enhanced intimal proliferation and marked impairment of endothelial vasodilator function occurred despite significant protection of the LDL particle against ex vivo copper-mediated oxidation.

These data indicate that in vitro assay of LDL "protection" against oxidative damage is problematic. The primary site of LDL modification in vivo is likely to be the vascular wall (33). Ascorbate (23, 47) and urate (48) effectively limit significant

LDL modification in plasma, and ox-LDL that may form in plasma is rapidly cleared by the liver (49). Ex vivo assays of LDL oxidation are limited in that only the intrinsic properties of the LDL particle are of importance. Potentially significant effects of antioxidants on tissue-mediated LDL modification are not addressed by this assay. For example, probucol is a lipid-soluble antioxidant that is incorporated into the LDL particle and affords significant protection against ex vivo copper-mediated oxidation (50). However, cells loaded with probucol demonstrate significant impairment in their ability to modify LDL (51) suggesting an alternative intracellular site of action. Similarly, Navab and colleagues (34) have demonstrated that endothelial cell/smooth muscle cell co-cultures preincubated with probucol, $\alpha$-tocopherol, or $\beta$-carotene demonstrate a diminished capacity to modify LDL. Thus, our observation of continued LDL protection against ex vivo copper-mediated oxidation in the face of accelerated intimal proliferation and impaired endothelial function may reflect the inadequacy of this parameter as an assay of vascular "oxidative state."

Alternatively, our results may be explained by $\alpha$-tocopherol-mediated enhancement of LDL oxidation and, thus, increased EDRF inactivation and/or endothelial cell toxicity. It seems unlikely that $\alpha$-tocopherol itself is directly cytotoxic as prolonged incubation of normocholesterolemic vessels with $\alpha$-tocopherol did not result in impaired endothelium-dependent arterial relaxation (Fig. 4). Bowry and colleagues (52) have demonstrated enhanced LDL oxidation by aqueous peroxyl radicals mediated by high levels of $\alpha$-tocopherol in LDL. These authors suggested that at slow rates of radical generation such as those observed in vivo the $\alpha$-tocopheroxyl radical formed in LDL may act as a radical chain propagating, rather than terminating agent, and may also introduce aqueous radicals into the LDL particle (52). Recent observations that nitric oxide (or its metabolites) may react with $\alpha$-tocopherol to form the tocopheroxyl radical may also have important implications for enhanced LDL oxidation (53, 54). Observations by Godfried and colleagues (28) that excess $\alpha$-tocopherol promotes the development of intimal proliferation would seem to support these findings and are consistent with our results. Our LDL oxidation studies did not demonstrate enhanced LDL susceptibility to oxidation in the high-dose $\alpha$-tocopherol group because the pro-oxidant activity of $\alpha$-tocopherol is only observed with a metal-ion independent type of oxidative stress, but not with copper ions (52).

In summary, the results presented here demonstrate that dietary treatment with modest doses of the lipid-soluble antioxidant, $\alpha$-tocopherol, preserves endothelium-dependent vasorelaxation in cholesterol-fed rabbits without any significant effect on intimal proliferation. Plasma $\alpha$-tocopherol levels in animals from the low-dose $\alpha$-tocopherol group approximated those found in patients receiving 1,200 IU of $\alpha$-tocopherol supplementation daily (15). In this study, higher doses of $\alpha$-tocopherol were associated with markedly impaired endothelium-dependent arterial relaxation and enhanced intimal proliferation compared to rabbits fed only cholesterol. In both instances, LDL $\alpha$-tocopherol content was enhanced and was associated with increased LDL resistance to ex vivo copper-mediated oxidation. With respect to human hypercholesterolemia and atherosclerosis, these results should be interpreted with some caution as the plasma levels of vitamin E reported here exceed those achievable in man. Nevertheless, these observations suggest that excess dietary $\alpha$-tocopherol is potentially harmful and that assay of ex vivo copper-mediated LDL oxidation may be a poor reflection of in vivo protection against oxidative damage.

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