The effects of high levels of vitamin E on the progression of atherosclerosis in the Watanabe heritable hyperlipidemic rabbit

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Watanabe rabbits (5–6 months of age) were divided into two groups (seven each). One group was fed standard Purina Rabbit Chow and the second was fed the same diet supplemented with 2000 mg/kg diet vitamin E. (Purina Rabbit Chow contains about 30 mg vitamin E/kg diet). After 9 months, the rabbits were sacrificed and plasma lipids were determined, and the area of plaque involvement in the aortas was measured. The extent of plaque formation (percentage of the total surface covered with fatty streaks) was not significantly different in the two groups. However, there were differences observed in plasma lipids. Plasma total cholesterol, low density lipoprotein cholesterol, and triglyceride levels were 20–30% lower in the vitamin E-fed group. Also, the concentration of cholesterol in the lesions of the aortic arch was about 25% lower in the vitamin E-fed group. These results indicate that high levels of vitamin E may have a beneficial effect on lowering plasma lipids observed in hyperlipidemia. (J. Nutr. Biochem. 4:651–654, 1993.)

Keywords: hypercholesterolemia; hyperlipidemia; vitamin E; α-tocopherol; LDL cholesterol; WHHL rabbits

Introduction

Several groups have demonstrated that probucol, a hypocholesterolemic drug, acts as an antioxidant to prevent the progression of atherosclerosis in the Watanabe heritable hyperlipidemic rabbit (WHHL).[1–3] We initiated a study to determine whether vitamin E, a naturally occurring antioxidant, might act similarly in these heritable hyperlipidemic rabbits. The antioxidant properties of vitamin E are well known, and the potential of vitamin E as a therapeutic drug used to treat atherosclerosis has been suggested.[4] Although how antioxidants prevent atherosclerosis is not known, data suggest that vitamin E prevents oxidation of low density lipoproteins (LDL).[5–7] Evidence proposing a role for vitamin E in prevention and reversal of diet-induced atherosclerosis in rabbits and monkeys has been published.[8–10] However, one study indicated a potentiating effect of vitamin E on rabbits fed an atherogenic diet.[11] To investigate further the antiatherogenic properties of vitamin E, we tested the effect of high levels of dietary vitamin E on the progression of atherosclerosis in Watanabe rabbits. While our study was being completed, results from a similar study were published by another group.[12] Although our results generally corroborate theirs, differences in animals, levels of vitamin E, and experimental design may have been responsible for some differences in our results, which are reported here.

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Methods and materials

Animals and diets

Homozygous 5–6-month-old male Watanabe rabbits were purchased from the National Institutes of Health (NIH), Bethesda, MD USA. Animals were divided into two groups of seven and fed either standard rabbit diet (Purina, St. Louis, MO USA) or the same diet supplemented with α-tocopherol-acetate (Sigma Chemical Co., St. Louis, MO USA) at 2000 mg/kg of diet (0.2%). At the onset of this study, it was only possible to obtain seven 5-mo- and seven 6-mo-old Watanabe rabbits from NIH. They were divided so that the fed group

contained four 5-mo-old and three 6-mo-old rabbits. The control group comtained three 5-mo-old and four 6-mo-old rabbits. Because the major objective was to measure the progression of atherosclerosis in the presence or absence of vitamin E for comparison with the effect of probucol in similar experiments;[1] a single 9-month period was chosen. Average intake of diet was 150 g/day/rabbit. Thus, the average intake of vitamin E in the experimental group was 300 mg/day/rabbit.

Plasma lipid and vitamin E analysis

At the end of the 9-mo experimental period, animals were sacrificed by sodium pentobarbital overdose. The thoracic and abdominal cavities were opened and a heparinized blood sample was taken by heart puncture prior to perfusion with buffer.[2] Plasma was collected by centrifugation for lipid and vitamin E analysis. The aorta was surgically removed, trimmed of non-aortic tissue, and divided into three sections (aortic arch, thoracic, and abdominal sections) for determination of lesion development. Total cholesterol, LDL cholesterol, and triglyceride levels were determined using commercially available kits (LDL-Direct Plus Cholesterol System, IsoLab, Inc., Akron, OH USA; Triglyceride GPO-Trinder, Sigma Chemical Co.) according to the recommendations of the manufacturers. The cholesterol assay is based on the method of Bentzen et al.,[13] which separates the α-lipoprotein (high density lipoprotein) and β-lipoproteins (LDL and very LDL) by affinity chromatography. Total cholesterol and the cholesterol in each fraction were determined by the manufacturer's enzymatic reagent system. Only the values for total and β-lipoprotein fractions (referred to as LDL) are reported. When the cholesterol content of α- and β-lipoprotein fractions were added together and compared with total cholesterol, the recovery was 103 ± 2% for all samples. The triglyceride assay is a modification of the method of McGowan, et al.[14] Vitamin E levels were determined by high performance liquid chromatography (HPLC) essentially as described elsewhere.[15] Plasma samples were divided into two 1-mL aliquots in 12 × 1.5 cm screw cap Pyrex tubes. To one sample we added 1 mL ethanol, and to the second 1 mL ethanol containing 5 μg α-tocopherol for the purpose of quantitation. The tubes were mixed vigorously to precipitate proteins. Then, 5 mL of hexane was added, the tubes were shaken for 1 min, and the layers were allowed to separate. Four mL of the hexane layer was removed for analysis of α-tocopherol. α-Tocopherol was separated from the high levels of lipids in these plasma samples by thin layer chromatography (TLC) using silica gel G in a solvent of hexane, acetic acid, and diethyl ether, 73:2:25 (vol/vol/vol). The R_f values for cholesterol ester, triglycerides, α-tocopherol, cholesterol, and phospholipids were about 1.0, 0.7, 0.6, 0.3, and 0, respectively. The hexane extract of the plasma sample was evaporated to dryness under nitrogen, and the residue was dissolved in 0.2 mL hexane that was applied to the TLC plate and chromatographed. The area of the gel containing the α-tocopherol (visualized by iodine) was scraped off the plate and eluted with diethyl ether. The ether was evaporated under nitrogen and the residue dissolved in 100 μL hexane. A 25 μL aliquot was injected onto the HPLC column (3.9 × 300 cm, C18, 125A, 10 μm, Waters, Milford, MA USA) using a Spectra Physics HPLC system. Elution was in methanol and water 97:3 (vol/vol). α-Tocopherol was quantitated by measuring the OD at 280 nm and comparison of peak heights of samples with or without added α-tocopherol (5 μg). Because the plasma samples from the Watanabe rabbits (controls and vitamin E-treated) contained such high levels of lipids that direct HPLC on the hexane extract could not achieve quantitation of α-tocopherol, TLC to remove most lipids was necessary. Although this lowered the recovery, the reproducibility was good. After addition of 5 μg internal standard, recovery of α-tocopherol from normal rabbit plasma (not hyperlipidemic) assayed directly by HPLC of the hexane extract was 87.4 ± 5.5% (± SEM, n = 3). Recovery after TLC from hexane extracts of hyperlipidemic rabbit plasma was 44.3 ± 2.4% (± SEM, n = 3). The presence of other forms of tocopherols in these samples was not determined.

Determination of aortic lesions and cholesterol content

The percent surface area containing fatty streak lesions was determined as previously described.[2] Aortas were divided into three sections, the aortic arch, the thoracic aorta, and the abdominal aorta. The aortic arch and thoracic aorta were divided between the origin of the left subclavian artery and the first intercostal artery. The thoracic and abdominal aortas were divided 5 mm proximal to the celiac artery. The aortas were opened longitudinally, pinned flat on wax sheets, fixed overnight in modified Karnovskys solution as described[16] (2% paraformaldehyde, 1.5% glutaraldehyde, 2.5 mM $CaCl_2$, 0.1 M sodium cacodylate buffer, pH 7.2), and photographed. The photographs were enlarged (2 ×) and enhanced with high contrast filters so that the fatty lesions could be quantitated by computer-assisted planimetry (Bioquant, R and M Biometrics, Inc., Nashville, TN USA). After photographing the fixed and stained tissues, samples were blotted dry on filter paper, weighed, and placed in 12 × 1.5 cm screw cap Pyrex tubes. The tissues were digested by incubating in 1N KOH, 67% ethanol (0.1 mL/mg tissue) for 3 hr at 100° C. Total cholesterol was determined in 30 μL aliquots of the digest by the same method used for plasma samples. The small amount of alcoholic KOH did not inhibit the assay.

Statistical analysis

Statistical comparisons were made between vitamin E-fed and control rabbits using Student's *t* test.

Results

Plasma lipid and vitamin E levels are shown in *Table 1*. There was a clear tendency toward lowering of plasma LDL cholesterol, total cholesterol, and triglyceride in response to a high vitamin E diet. There were no significant differences observed in the percentage area of aortic lesions as a result of high level of vitamin E (*Table 2*), which is different from the results of Williams et al.[12] This could be due to the fact that we used purebred WHHL rabbits that were already 5–6 mo old at the start of the experiment. One extra WHHL rabbit was sacrificed at the beginning of the experiment and the percentage area of lesion determined. This rabbit already contained 37% lesion development in the aortic arch. In comparison with the study of Williams et al.,[12] our experiments were conducted over 9 months rather than 12 weeks, and a lower amount of vitamin E was added to the diet. However, when the cholesterol content of these tissues was determined there was a lower amount in the aortic arch segment (which contained the most lesions) in rabbits fed high vitamin E (*Table 3*). These results suggest that although lesions covered similar amounts of arterial surface in control and treated

Table 1 Plasma levels of LDL, total cholesterol, triglycerides, and vitamin E

	Concentration		
	Standard diet	Vitamin E diet	Significance
LDL cholesterol (mmol/L)	5.79 ± 0.39	4.32 ± 0.59	$P < 0.09$
Total cholesterol (mmol/L)	8.48 ± 0.36	6.90 ± 0.77	$P < 0.10$
Triglycerides (mmol/L)	6.37 ± 1.25	4.29 ± 0.53	$P < 0.17$
Vitamin E (μmol/L)	93 ± 46	497 ± 142	$P < 0.01$

Values are expressed as the mean ± SEM

rabbits, the lesions were not as advanced in the vitamin E-fed group.

Discussion

These experiments were designed to test the effect of vitamin E, a naturally occurring antioxidant, on the progression of aortic lesions and on plasma lipid in the Watanabe rabbit. These rabbits are deficient in LDL receptor and develop hypercholesterolemia and atherosclerosis while being fed a normal rabbit diet. Thus, these rabbits are considered to be a better model to compare with familial hypercholesterolemia in humans than normal rabbits fed an atherogenic, hypercholesterolemic diet. Results from these studies suggest that high dietary vitamin E reduces plasma cholesterol and triglycerides. Although the mean values were decreased by 20–30%, there was enough variation between animals in each group to cause P values to be of borderline statistical significance. This was not due to the lack of precision of the assays, but may have been due to lack of proper distribution of littermates between the two groups.[17] The colorimetric assays varied by less than 2% when the same samples were assayed on different days. The statistical evaluation of the data indicates that there was greater than a 90% (but not 95%) probability that the vitamin E-fed and control-diet rabbits have different levels of total cholesterol and LDL cholesterol in plasma and total cholesterol in the aortic arch lesions. The observation of less cholesterol in arch lesions is probably due to a difference in thickness because the surface arch covered by lesions was not significantly different. Although statistical significance was not obtained in any variable other than vitamin E, we feel that these results support other published data[12] and may provide a basis for a more rational approach to similar experiments in the future. Differing from that report, we did not see differences in the percentage area of lesion development in the aorta. However, the vitamin E-fed group possessed a lower concentration of cholesterol in the aortic arch, which contained the highest percentage area of lesions. The differences between our study and Williams et al.[12] are that they used a modified Watanabe rabbit,[18] and we used purebred Watanabe rabbits, which probably contained significant lesion development at the onset of the study. Also, we fed a lower amount of vitamin E (0.2% versus 0.5%) for a longer period of time (9 mo versus 12 wks). However, the plasma levels of vitamin E were similar in both studies, suggesting a saturation of plasma levels of vitamin E at lower dietary levels.

There was no significant difference observed between the levels of HDL in the two groups (data not shown). One report indicates that in some cases HDL is increased in humans fed megadoses of vitamin E.[19] Although the mechanism by which vitamin E may lower LDL cholesterol in these rabbits is not known, it may be similar to the mechanism proposed for probucol.[20] That proposal suggests that probucol induces alterations in LDL structure, thus enhancing LDL removal from the plasma. These alterations in LDL particles have

Table 2 Aortic lesion development

	Aortic surface area lesion development (percent)	
	Standard diet	Vitamin E diet
Aortic arch	71 ± 8	68 ± 15
Thoracic aorta	18 ± 19	19 ± 24
Abdominal aorta	12 ± 8	27 ± 28

Values are expressed as the mean ± SEM

Table 3 Total cholesterol extracted from aortal tissue

	Total cholesterol (mg/g tissue wet wt.)		
	Standard diet	Vitamin E diet	Significance
Aortic arch	20.0 ± 2.2	14.8 ± 0.7	$P < 0.09$
Thoracic aorta	5.5 ± 1.4	5.2 ± 2.6	NS
Abdominal aorta	2.2 ± 0.7	3.3 ± 0.2	NS

Values are expressed as the mean ± SEM
NS, not significant.

been proposed to be due to the antioxidant properties of probucol,[1,2] which may also be the case for vitamin E.

In conclusion, these results support a hypocholesterolemic and a hypotriglyceridemic effect of high dietary vitamin E, which may be beneficial in the treatment of hyperlipidemia in humans that, like the WHHL rabbit, are deficient in LDL receptors. Although the data are somewhat variable, we feel that their presentation is worthwhile because the observed trends support other data relating to the possible importance of the effect of vitamin E on hyperlipidemia and atherosclerosis.

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