Palm Tocotrienols Protect ApoE +/− Mice from Diet-Induced Atheroma Formation

Tracy M. Black,*† Ping Wang,† Nobuyo Maeda** and Rosalind A. Coleman†2

Departments of *Medicine, **Pathology and †Nutrition, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599

ABSTRACT We evaluated the effects of vitamin E and β-carotene on apolipoprotein (apo)E +/− female mice, which develop atherosclerosis only when fed diets high in triglyceride and cholesterol. Mice were fed a nonpurified control diet (5.3 g/100 g triglyceride, 0.2 g/100 g cholesterol), an atherogenic diet alone (15.8 g/100 g triglyceride, 1.25 g/100 g cholesterol, 0.5 g/100 g Na cholate) or the atherogenic diet supplemented with either 0.5 g/100 g (+)-α-tocopherol (mixed isomers); 0.5 g/100 g palm tocopherols (palm-E; 33% α-tocopherol, 16.1% α-tocotrienol, 2.3% β-tocotrienol, 32.2% γ-tocotrienol, 16.1% δ-tocotrienol); 1.5 g/100 g palm-E; or 0.01 g/100 g palm-carotenoids (58% β-carotene, 33% α-carotene, 9% other carotenoids). Compared with mice fed the control diet, plasma cholesterol was fourfold greater in mice fed the atherogenic diet. Mice fed the 1.5 g/100 g palm-E supplement had 60% lower plasma cholesterol than groups fed the other atherogenic diets. Mice fed the atherogenic diet had markedly higher VLDL, intermediate density lipoprotein (IDL) and LDL cholesterol and markedly lower HDL cholesterol than the controls. Lipoprotein patterns in mice supplemented with α-tocopherol or palm carotenoids were similar to those of the mice fed the atherogenic diet alone, but the pattern in mice supplemented with 1.5 g/100 g palm-E was similar to that of mice fed the control diet. In mice fed the atherogenic diet, the hepatic cholesterol plus cholesterol ester concentration was 4.4-fold greater than in mice fed the control diet. Supplementing with 1.5 g/100 g palm-E lowered hepatic cholesterol plus cholesterol ester concentration 66% compared with the atherogenic diet alone. Mice fed the atherogenic diet had large atherosclerotic lesions at the level of the aortic valve. With supplements of 0.5 g/100 g palm-E or 1.5 g/100 g palm-E, the size of the lesions was 92 or 98% smaller, respectively. The 0.5 g/100 g α-tocopherol and palm carotenoid supplements had no effect. Supplements did not alter mRNA abundance for apolipoproteins A1, E, and C3. The beneficial effect of tocotrienols on atherogenesis, the plasma lipoprotein profile and accumulation of hepatic cholesterol esters cannot be attributed to their antioxidant properties.

KEY WORDS: • antioxidants • β-carotene • vitamin E • atherosclerosis • apoE gene knockout mice

Atherosclerosis is a major cause of morbidity and mortality in nations with Western lifestyles. Although the development of atherosclerosis has been linked to hypercholesterolemia, the formation of an atherosclerotic plaque is not simply the accumulation of cholesterol and cholesterol esters within arterial walls; instead, it is a complex dynamic process involving mechanisms that include release of chemotaxis factors and cytokines from endothelial cells, chemotaxis and migration of monocytes into the subendothelial space, migration and proliferation of smooth muscle cells and apoptosis (Berliner 1995, Ross 1993, Witztum 1994). The oxidation of lipoproteins is believed to play a major role in stimulating the processes that lead to plaque formation; however, it has not been determined directly whether preventing lipoprotein oxidation will decrease atherosclerotic plaque formation. Further, it has not been determined whether the proposed effects of vitamin E or β-carotene on atherosclerosis are due to antioxidant properties of these vitamins. Epidemiologic studies and studies in animal models of atherosclerosis have shown variable responses to vitamin E and β-carotene. Vitamin E supplements are positively associated with a lower risk of atherosclerotic disease (Rimm et al. 1993, Stampfer et al. 1993), but intervention studies with antioxidants have provided conflicting results. In human studies, supplementation with α-tocopherol resulted in fewer myocardial events or deaths (Stampfer et al. 1993, Stephens et al. 1996), but benefits from β-carotene have not been shown (Hennekens et al. 1996, Omenn et al. 1996). Animal studies with α-tocopherol have been similarly inconclusive (Upston et al. 1999). The antioxidant Probucol decreases atherosclerotic lesion formation in Wantanabe heritable hyperlipidemic rabbits (Carew et al. 1987), but paradoxically increases lesion formation in apolipoprotein E (apoE)1 knockout mice (Zhang et al. 1997),

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2 To whom correspondence should be addressed.

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whereas the antioxidant, N,N'-diphenyl-1,4-phenylenediamine, decreases aortic plaque formation in these mice (Tangirala et al. 1995). In other studies of homozygous apoE knockout mice, 0.05 g/100 g α-tocopherol or 0.05 g/100 g β-carotene had no effect on the spontaneous formation of atherosclerotic lesions (Shaish et al. 1999), whereas 2000 it/kg vitamin E (~0.2 g/100 g) reduced the progression of atherosclerotic lesions (Pratico et al. 1998). Of the carotenoids, all-trans-β-carotene but not 9-cis-β-carotene decreased atherogenic plaques in New Zealand White rabbits fed an atherogenic diet (Shaish et al. 1995).

To test the effects of vitamin E and β-carotene on the development of atherosclerotic lesions in a systematic manner, we used adult female apoE +/- mice, which are deficient in apoE, the high affinity ligand for the LDL receptor (Weisgraber 1994). ApoE is the major ligand for removing cholesterol-apoE, the high affinity ligand for the LDL receptor (Weisgraber et al. 1994). ApoE is the major ligand for removing cholesterol-rich chylomicra and VLDL remnants from the blood. Unlike homozygous apoE --/-- mice, which develop atherosomas when fed a rodent nonpurified diet, heterozygous apoE +/-- mice develop atherosclerotic lesions only when they are fed a diet that is high in saturated fat and cholesterol (Zhang et al. 1994).

MATERIALS AND METHODS

**Animals.** Animal protocols were approved by the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee. ApoE +/- female mice were bred on site from adult mouse C57BL/6J (B6) mice (Charles River Laboratory, Wilmington, MA) and male homoygous apoE --/-- mice. The male apoE --/-- mice were the result of backcrossing B6:129F1 heterozygotes (Piedrahita et al. 1992) to B6 for six generations before intercrossing to obtain the apoE +/- heterozygotes. The mice were maintained on a 12-h light:dark cycle. After weaning, the pups had free access to a nonpurified diet until they were 8–12 wk old (see below). Females, in groups of 4, were then fed one of six diets (see below) for 3 mo. The mice were weighed at the start (d 0) and the completion (d 90) of the experimental diets. At the beginning of the study, each group contained 16 mice except the 1.5 g/100 g palm-E group which contained 14. Deaths in each group occurred in association with anesthesia used during blood drawing. There were 3 deaths in the atherogenic group, 1 in the 0.5 g/100 g palm-E group, 2 in the 1.5 g/100 g palm-E group, and 1 in the α-tocopherol group.

**Diets.** The nonpurified diet (Prolab RMH 3000, Purina Mills, St Louis, MO) contained 5.3 g/100 g triglyceride and 0.2 g/100 g cholesterol. The atherogenic diets were prepared by Dyets (Bethlehem, PA) to contain 18.5 g/100 g triglyceride, 1.25 g/100 g cholesterol and 0.5 g/100 g sodium cholate and supplemented with either 0.5 g/100 g (+) -α-tocopherol (mixed isomers; Sigma Chemical, St Louis, MO); 0.5 g/100 g mixed palm-tocopherols (palm-E) (33% α-tocopherol, 16.1% α-tocotrienol, 23% β-tocotrienol, 32.2% γ-tocotrienol, 16.1% β-tocotrienol); 1.5 g/100 g palm-E; or 0.01 g/100 g mixed palm carotenoids (58% β-carotene, 33% α-carotene, 9% other carotenoids). The palm-E supplements were added at 0.5 g/100 g to be equivalent to vitamin E in the α-tocopherol supplement, and at 1.5 g/100 g, which contains 0.5 g/100 g α-tocopherol. The palm-derived supplements were kindly supplied by Dr. Kalyana Sundram of the Palm Oil Research Institute of Malaysia. The mice had free access to food and water during the study period.

**Lipid analyses.** Plasma for total cholesterol and triglyceride was obtained at d 0 and 90 of the study. The mice were deprived of food overnight, then anesthetized with 0.2–0.4 mL of 20 g/L tri bromoethanol (Avertin, Aldrich Chemical, Milwaukee, WI). Heparinized whole blood was placed in 1.5-mL Eppendorf tubes containing 10 μL of 10 mmol/L EDTA. The blood was then centrifuged for 10 min at 12,000 × g at 4°C. Plasma was assayed for triglyceride (GPO-Trinder kit, #339–20, Sigma Chemical) and cholesterol (Cholesterol CII kit, #276–64909, Wako Pure Chemical, Osaka, Japan) with colorimetric enzyme methods.
of the standard deviations among the groups. Data for liver cholesterol, triglyceride and phospholipid were analyzed using the General Linear Models procedure of SAS, appropriate for a completely randomized design (SAS 1989). Treatment differences were assessed using a protected Least Significant Difference test (Steel et al. 1997) at $P_{0.05}$.

**RESULTS**

**Plasma lipids.** Mouse weights did not differ among groups at 90 d ($P = 0.28$) (Table 1). Although plasma triglyceride concentrations were lower in most of the atherogenic diet groups compared with controls, the differences were not significant. In mice fed the atherogenic diet, plasma cholesterol concentration was fourfold greater than in mice fed the control diet. When the atherogenic diet was supplemented with $\alpha$-tocopherol, palm carotenoids or 0.5 g/100 g palm-E, plasma cholesterol tended to be an additional 20–33% greater. In contrast, compared with mice fed the atherogenic diet alone, plasma cholesterol was 58% lower when mice were fed the atherogenic diet supplemented with 1.5 g/100 g palm-E (Table 1).

**FPLC analysis of lipoproteins.** When apoE $+/-$ mice were fed the nonpurified control diet, the major lipoprotein peak was HDL (fractions 17–24) (Fig. 1). The atherogenic diet changed this pattern radically, i.e., the major cholesterol-containing fractions were VLDL (fractions 3–6), intermediate density lipoprotein (IDL) (fractions 6–8), and LDL (fractions 8–16), and the HDL peak decreased. Supplementation with palm carotenoids had no effect on this pattern but 0.5 g/100 g $\alpha$-tocopherol appeared to decrease the LDL peak and the 0.5 g/100 g palm-E supplements appeared to decrease both IDL and LDL peaks, although sufficient samples were not available for statistical analysis. In mice supplemented with 1.5 g/100 g palm-E, however, the VLDL, IDL and LDL peaks were mark-

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**TABLE 1**

Weight and changes in plasma triglyceride (TG) and plasma cholesterol in apolipoprotein E (apoE) $+/-$ female mice fed control and atherogenic diets with and without supplements for 90 d$^{1,2}$

<table>
<thead>
<tr>
<th>Diet</th>
<th>Final weight g</th>
<th>Change in TG $^3$ mg/dL</th>
<th>TG final 30.4 mg/dL</th>
<th>Change in cholesterol $^4$ mg/dL</th>
<th>Cholesterol final 62 mg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonpurified control</td>
<td>21.2 ± 2.3</td>
<td>-3.8 ± 11.9</td>
<td>30.4 ± 4.4</td>
<td>0.12 ± 35.4</td>
<td>62 ± 31</td>
</tr>
<tr>
<td>Atherogenic</td>
<td>20.7 ± 1.7</td>
<td>-9.3 ± 16.1</td>
<td>25.4 ± 4.2</td>
<td>244 ± 87$^b$</td>
<td>300 ± 97</td>
</tr>
<tr>
<td>Atherogenic + 0.5 g/100 g $\alpha$-tocopherol</td>
<td>22.3 ± 1.4</td>
<td>-11.8 ± 16.2</td>
<td>24.3 ± 7.6</td>
<td>323 ± 172$^b$</td>
<td>402 ± 167</td>
</tr>
<tr>
<td>Atherogenic + 0.01 g/100 g palm-carotenoids</td>
<td>21.7 ± 1.3</td>
<td>-16.7 ± 14.8</td>
<td>23.1 ± 5.7</td>
<td>301 ± 118$^b$</td>
<td>362 ± 108</td>
</tr>
<tr>
<td>Atherogenic + 0.5 g/100 g palm-tocopherols</td>
<td>21.5 ± 2.3</td>
<td>-1.1 ± 13.9</td>
<td>25.8 ± 3.1</td>
<td>321 ± 122$^b$</td>
<td>397 ± 116</td>
</tr>
<tr>
<td>Atherogenic + 1.5 g/100 g palm-tocopherols</td>
<td>20.1 ± 1.3</td>
<td>5.2 ± 9.1</td>
<td>26.3 ± 8.3</td>
<td>51 ± 53$^a$</td>
<td>126 ± 41</td>
</tr>
</tbody>
</table>

$^1$ Data are presented as means ± SD. For all initial groups, $n = 16$, except for 1.5 g/100 g palm-tocopherol, $n = 14$; for final groups, $n = 17$ for $\alpha$-tocopherol, $n = 16$ for nonpurified diet, $n = 13$ for atherogenic, $n = 15$ for 0.5 g/100 g palm-tocopherols, and $n = 12$ for 1.5 g/100 g palm-tocopherols. Superscripts indicate significant differences between changes in triglyceride and cholesterol in atherogenic diet groups ($P < 0.05$) by ANOVA with Scheffé’s test. (Differences in final concentrations were not analyzed).

$^2$ Changes in groups fed the atherogenic diet plus supplements are compared to mice fed the atherogenic diet alone.

$^3$ Conversion factor for triglyceride = 0.01129 for mmol/L.

$^4$ Conversion factor for cholesterol = 0.02586 for mmol/L.

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**FIGURE 1** Cholesterol concentrations (arbitrary units) in lipoprotein fractions (0.5 mL) from apolipoprotein E (apoE) $+/-$ female mice fed control and atherogenic (Ath) diets supplemented with 0.5 g/100 g palm-tocopherols (palm-E), 1.5 g/100 g palm-E, 0.5 g/100 g $\alpha$-tocopherol, or 0.01 g/100 g palm carotenoids. Serum fractions were separated by Superose 6 column fast performance liquid chromatography. The analyses were performed in duplicate using two combined serums samples, $n = 4/diet$ group. IDL, intermediate density lipoprotein.
supplemented with palm carotenoids. Lesion size and thickness were diminished in the mice supplemented with 0.5 g/100 g palm-E, and the aortic histology in mice supplemented with 1.5 g/100 g palm-E was virtually indistinguishable from that of heterozygote mice fed the nonpurified control diet.

**Hepatic lipid.** Because the livers of the mice fed the atherogenic diet alone appeared pale and fatty, whereas those of mice consuming the atherogenic diet plus 1.5 g/100 g palm-E appeared normal, we examined liver histology and analyzed liver lipids. Oil red O, which interacts with neutral lipids such as triglyceride and cholesteryl esters, stained numerous lipid droplets in livers from mice fed the atherogenic diet and fewer droplets from the mice supplemented with 1.5 g/100 g palm-E (Fig. 4). In the 1.5 g/100 g palm-E group, lipid droplets were well formed, discrete and smaller. The un-supplemented atherogenic group had large lakes of lipid accumulation with clefts suggestive of cholesterol crystals. The cholesterol content of the droplets was confirmed by chemical analysis. In mice fed all of the atherogenic diets, hepatic triglyceride concentration was ~30% lower than in mice fed the nonpurified control diet. The phospholipid concentration in liver did not differ among the groups (Table 2). In contrast, the atherogenic diet caused the concentration of liver free cholesterol plus cholesteryl ester to increase 4.4-fold. Supplemented palm carotenoids, a-tocopherol, and 0.5 g/100 g palm-E had no effect on the high liver cholesterol concentration produced by the atherogenic diet; however, the 1.5 g/100 g palm-E supplement reduced the hepatic concentration of cholesterol plus cholesteryl ester 66%.

**Hepatic expression of mRNA of selected enzymes and apolipoproteins.** No significant differences were observed in liver mRNA abundance of apolipoproteins A1, E and C3 (data not shown).

**DISCUSSION**

When an atherogenic diet was fed to apoE +/- female mice and supplemented with a mixture of vitamin E compounds derived from palm oil, the formation of atherosclerotic lesions was attenuated substantially. Supplementation with a-tocopherol or palm-carotenoids had no effect. Of the diets that contained palm-E, the more striking effect occurred in the 1.5 g/100 g palm-E group. This effect was probably due to the content of tocotrienols in the supplement, which contained as much a-tocopherol (33%) as was present in the 0.5 g/100 g a-tocopherol supplement. Moreover, the 0.5 g/100 g palm-E supplement was more effective than the 0.5 g/100 g a-tocopherol supplement. Tocotrienols are similar in structure to tocopherols, but contain three double bonds in the isoprenoid chain. In addition to their antioxidant properties (Kamal-Eldin and Appelqvist 1996), tocotrienols lower total and LDL cholesterol in hamsters (Khor and Chiang 1996) and rats (Watkins et al. 1993). Tocotrienols have been shown to decrease serum cholesterol in some (Qureshi et al. 1991, 1995 and 1997), but not all (Mensink et al. 1999, Tan et al. 1991) human studies. These reported effects on serum cholesterol may have occurred because tocotrienols suppress 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase activity and decrease the secretion of apoB. Tocotrienols both decrease translation of HMG-CoA reductase mRNA (Parker et al. 1993) and increase degradation of the reductase protein (Pearce et al. 1992). They also increase proteosome-mediated degradation of apoB, the most lipoprotein secreted in VLDL (Wang et al. 1998). However, the effects on HMG-CoA reductase that would lead to decreased synthesis and secretion of VLDL cholesterol are unlikely to be relevant to this study. The apoE

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**FIGURE 2.** Morphometric evaluation of atherosclerotic lesion size at the level of the aortic sinus of apolipoprotein E (apoE) +/- female mice fed an atherogenic diet with or without supplements of 0.5 g/100 g palm-tocopherols (palm-E), 1.5 g/100 g palm-E, 0.5 g/100 g a-tocopherol, or 0.01 g/100 g palm carotenoids for 90 d. Each point represents the mean lesion size of four sections measured in each mouse (n = 8, no supplement; n = 3, 0.5 g/100 g palm E; n = 9, 1.5 g/100 g palm E; n = 4, 0.5 g/100 g α-tocopherol; n = 14, palm carotenoids). Note that the vertical axis is logarithmic. The horizontal bars represent the logarithmic means in μm² of lesion size from each group of mice. Letters indicate significant differences between groups analyzed by ANOVA with Scheffe’s test (P < 0.05). Mice fed the control diet (not shown) for 90 d had no atherosclerotic lesions. At the end of the study, mice were 20–22 wk old.

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**TABLE 2.** Hepatic expression of mRNA of selected enzymes and apolipoproteins. No significant differences were observed in liver mRNA abundance of apolipoproteins A1, E and C3 (data not shown).
1/2 mice were fed atherogenic diets that contained a high amount of cholesterol (1.25 g/100 g), and the mice had a large excess of cholesterol stored in their livers. Excess hepatic cholesterol would be expected to suppress the proteolytic release of the nuclear transcription factor SREBP from the endoplasmic reticulum and minimize its entry into the nucleus and its action on gene transcription (Goldstein and Brown 1990). Thus, it is likely that de novo synthesis of cholesterol was already severely down-regulated in mice fed the atherogenic diets, and we would expect that little endogenous cholesterol synthesis would occur.

If tocotrienol effects on the already low HMG-CoA reductase do not provide a mechanism for attenuating either hepatic or serum hypercholesterolemia, what then accounts for the ability of the 1.5 g/100 g palm-E supplement to decrease hepatic cholesterol concentration and to normalize plasma lipoproteins? Possibilities include increased hepatic bile synthesis and secretion as well as reduced intestinal cholesterol absorption. Although not previously investigated, either of these possibilities might occur via tocotrienol-mediated effects that increase gene transcription of the 7-α-hydroxylase or that decrease bile acid and cholesterol transporters in the intestinal mucosa. Another possibility is that the pharmacologic amounts of tocotrienols present in the supplements might be preventing cholesterol absorption. Alternatively, increased degradation of apoB (Wang et al. 1998) might decrease plasma VLDL, LDL and cholesterol, although changes in apoB would not explain the lower hepatic cholesterol concentration.

The second remarkable finding of this study was the dramatic prevention of atheroma formation induced by the palm-E supplements. These results cannot be attributed to antioxidant effects alone. Vitamin E compounds are effective antioxidants because they can donate phenolic hydrogens to quench lipid free radicals. Although relative antioxidant potency has not been established, tocotrienols with their unsaturated side chain may be more mobile in membranes than tocopherols and thus better able to interact with lipid free radicals (Serbinova and Packer 1994). Others, however, have reported little difference between antioxidant potencies of tocopherols and tocotrienols (Kamal-Eldin and Appelqvist 1996). The mean lesion size for the 0.5 g/100 g α-tocopherol group was 3.7 times larger than that for the 0.5 g/100 g palm-E group. Because the diet of the 0.5 g/100 g palm-E group...
Vitamin E Reduces Atheroma Formation in Apo E−/− Mice

LIVER CHOLESTEROL ESTER, TRIGLYCERIDE AND PHOSPHOLIPID IN APOLIPOPROTEIN E (apoE) +/− FEMALE MICE FED CONTROL AND Atherogenic Diets WITH OR WITHOUT SUPPLEMENTS FOR 90 D

<table>
<thead>
<tr>
<th>Diet group</th>
<th>Cholesterol2</th>
<th>Triglyceride3</th>
<th>Phospholipid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonpurified control</td>
<td>3.3 ± 0.6</td>
<td>30.2 ± 4.7</td>
<td>28.6 ± 2.2</td>
</tr>
<tr>
<td>Atherogenic</td>
<td>17.8 ± 2.5</td>
<td>20.0 ± 5.3</td>
<td>25.7 ± 3.6</td>
</tr>
<tr>
<td>+ 0.5 g/100 g α-tocopherol</td>
<td>14.5 ± 2.6</td>
<td>20.5 ± 0.5</td>
<td>24.6 ± 5.6</td>
</tr>
<tr>
<td>Atherogenic + 0.01 g/100 g palm-tocarnooids</td>
<td>18.6 ± 3.0</td>
<td>19.3 ± 2.2</td>
<td>26.4 ± 2.3</td>
</tr>
<tr>
<td>Atherogenic + 0.5 g/100 g palm-tocarnooids</td>
<td>19.1 ± 4.5</td>
<td>20.4 ± 1.8</td>
<td>26.4 ± 1.7</td>
</tr>
<tr>
<td>Atherogenic + 1.5 g/100 g palm-tocarnooids</td>
<td>6.0 ± 0.88*</td>
<td>23.0 ± 2.0</td>
<td>29.6 ± 1.4</td>
</tr>
</tbody>
</table>

1 Values are means ± so, n = 3 or 4. Treatment differences were assessed using a protected least significant difference test. * P < 0.0001 vs. atherogenic diet.

2 Conversion factor for cholesterol = 0.02586 for mmol/L.

3 Conversion factor for triglyceride = 0.01129 for mmol/L.

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LITERATURE CITED


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