Dietary Cosupplementation With Vitamin E and Coenzyme Q₁₀ Inhibits Atherosclerosis in Apolipoprotein E Gene Knockout Mice

Shane R. Thomas, Steven B. Leichtweis, Knut Pettersson, Kevin D. Croft, Trevor A. Mori, Andrew J. Brown, Roland Stocker

Abstract—Intimal oxidation of LDL is considered an important early event in atherogenesis, and certain antioxidants are antiatherogenic. Dietary coenrichment with vitamin E (VitE) plus ubiquinone-10 (CoQ₁₀), which is reduced during intestinal uptake to the antioxidant ubiquinol-10, CoQ₁₀(H₂) protects, whereas enrichment with VitE alone can increase oxidizability of LDL lipid against ex vivo oxidation. In the present study, we tested whether VitE plus CoQ₁₀ cosupplementation is more antiatherogenic than either antioxidant alone, by use of apolipoprotein E−deficient (apoE−/−) mice fed a high-fat diet without (control) or with 0.2% (wt/wt) VitE, 0.5% CoQ₁₀, or 0.2% VitE plus 0.5% CoQ₁₀ (VitE+CoQ₁₀) for 24 weeks. None of the supplements affected plasma cholesterol concentrations, whereas in the VitE and CoQ₁₀ groups, plasma level of the respective supplement increased. Compared with control, plasma from CoQ₁₀ or VitE+CoQ₁₀ but not VitE-supplemented animals was more resistant to ex vivo lipid peroxidation induced by peroxyl radicals. VitE supplementation increased VitE levels in aorta, heart, brain, and skeletal muscle, whereas CoQ₁₀ supplementation increased CoQ₁₀ only in plasma and aorta and lowered tissue VitE. All treatments significantly lowered aortic cholesterol compared with control, but only VitE+CoQ₁₀ supplementation significantly decreased tissue lipid hydroperoxides when expressed per parent lipid. In contrast, none of the treatments affected aortic ratios of 7-ketocholesterol to cholesterol. Compared with controls, VitE+CoQ₁₀ supplementation decreased atherosclerosis at the aortic root and arch and descending thoracic aorta to an extent that increased with increasing distance from the aortic root. CoQ₁₀ significantly inhibited atherosclerosis at aortic root and arch, whereas VitE decreased disease at aortic root only. Thus, in apoE−/− mice, VitE+CoQ₁₀ supplements are more antiatherogenic than CoQ₁₀ or VitE supplements alone and disease inhibition is associated with a decrease in aortic lipid hydroperoxides but not 7-ketocholesterol.


Key Words: antioxidant ■ atherogenesis ■ oxidation ■ α-tocopherol ■ ubiquinol ■ ubiquinone

The LDL oxidation theory of atherosclerosis proposes that oxidation of LDL lipid in the intima is an important early and proatherogenic event.1,2 In support of this theory, oxidized lipids3,4 and proteins5–8 are present in human lesions and lipoprotein-like particles isolated from them. For example, in advanced human atherosclerotic lesions, ≈ 5% of cholesteryl linolate (C18:2), the major oxidizable lipid in LDL, is oxidized and present primarily as hydroperoxides and respective alcohols9 and oxoderivatives.10 In addition, atherosclerotic lesions also contain oxysterols11 and F₂-isoprostanes,12,13 prostaglandin-like and nonenzymatic lipid oxidation products of arachidonate.14 However, these secondary lipid oxidation products are localized predominantly in foam cells12,15 and are present at a lower concentration compared with primary oxidation products of C18:2.4,13,16

Although present in lesions, the extent to which different oxidized lipids cause or promote atherogenesis remains unknown. Lipid hydroperoxides (LOOH), the primary lipid peroxidation products formed during the initial stage of lipoprotein oxidation,17 may contribute to oxidative modification of apolipoprotein B-100 of LDL in vitro by means of secondary reactions.18 Potential atherogenic activities of 5-cholesten-3-β-OL-7-one (7-ketocholesterol [7KC]) and F₂-isoprostanes also have been described in vitro. For example, 7KC is cytotoxic to vascular cells and can impair cholesterol efflux in macrophages,11 whereas 8-epi prostaglandin F₂α modulates platelet aggregation and is a smooth muscle cell constrictor.19,20

Given the oxidation theory, inhibitors of lipoprotein lipid oxidation are considered to be potential antiatherogenic compounds. Indeed, several antioxidants inhibit atherosclerosis in various animal models of the disease.1 However, not all antioxidants that inhibit in vitro lipid oxidation attenuate...
atherogenesis, for reasons largely unknown. Also, in Watanabe hyperlipidemic rabbits, prevention of aortic lipid peroxidation itself is not sufficient for inhibition of atherosclerosis, which suggests that antioxidants that attenuate atherosclerosis may do so by means of actions in addition to or independent of inhibition of lipoprotein oxidation.

Plasma lipoproteins contain several endogenous antioxidants with \( \alpha \)-tocopherol (vitamin E [VitE]) and ubiquinol-10 (CoQ\(_{10}\)H\(_2\)) that represent important modulators of lipid peroxidation. As the major antioxidant present in lipoprotein extracts, VitE is commonly thought to be antiatherogenic. However, outcomes of VitE intervention studies on atherosclerosis in experimental animals and cardiovascular disease in humans overall have been inconclusive, if not disappointing. Despite this, supplementation of apolipoprotein E-deficient mice (apoE\(^-/-\)) mice with 0.2% (wt/wt) VitE was recently reported to attenuate atherosclerosis significantly in aortic root and to decrease aortic content of F\(_2\)-isoprostanes.

Compared with VitE, few studies have examined antiatherogenic potential of CoQ\(_{10}\)H\(_2\). CoQ\(_{10}\) is used for dietary supplementation studies because it is stable and effectively converted into the antioxidant active CoQ\(_{10}\)H\(_2\) on intestinal supplementation studies because it is stable and effectively converted into the antioxidant active CoQ\(_{10}\)H\(_2\). As the major antioxidant present in lipoprotein extracts, VitE is commonly thought to be antiatherogenic.

Aortic Biochemistry

Pooled aortas were pulverized in liquid N\(_2\), resuspended in 1.5 mL of buffer A, and homogenized as described. A 50-\( \mu \)L aliquot of homogenate was added to an equal volume of 5% metaphosphoric acid for ascorbate analysis and a further 50 \( \mu \)L removed for protein determination with the bicinchoninic acid assay kit (Sigma). Remaining homogenate was extracted in 500-\( \mu \)L aliquots added to 2 mL of methanol and 10 mL of hexane. The sample was mixed vigorously for 1 minute and centrifuged at 4°C. The hexane fraction then was dried and lipids redissolved into 400 \( \mu \)L of isopropanol. This extract was analyzed for lipid-soluble antioxidants, NAD, CE, and LOOH by high-performance liquid chromatography (HPLC) as described. LOOH were measured as a marker of primary lipid peroxidation because it is the primary and major lipid oxidation product formed in lipoproteins from apoE\(^-/-\) mice undergoing oxidation. A previous study has shown that 70% of [\( \text{H}\)]-C18:2-OOH added to mouse aorta before pulverizing is recovered as the hydroperoxide and 30% recovered as the corresponding alcohol and that [\( \text{H}\)]-C18:2 added to aortas before workup is not converted to [\( \text{H}\)]-C18:2-OOH or [\( \text{H}\)]-C18:2-OH. To confirm the presence of LOOH, postcolumn chemiluminescence detection was used before and after borohydride treatment of samples. All compounds detected were quantified by peak area comparison with authentic standards run under identical conditions.

**Materials**

C18:2 and cholesteryl arachidonate (together called cholesteryl esters [CE]), nonesterified cholesterol (NEC), 5,6-cholen-3\B\B-19-diol (19-hydroxycholesterol), ascorbate, formalin, EDTA, glyceral, butylated hydroxytoluene and chloramphenicol were obtained from Sigma Chemical Co. 7KC was from Steraloids Inc (Wilton). VitE (RRR-\( \alpha \)-tocopherol) and CoQ\(_{10}\) were generous gifts from Henkel BioRad. DMSO was from Matheson, and calcium, magnesium, and chloride-free Dulbecco’s PBS (Sigma) was prepared from nanopure water and stored over chelating resin and kept frozen at \( -80°C \) for lipid and antioxidant analyses or diluted in 5% metaphosphoric acid (1:1 vol/vol) and stored at \(-80°C\) for ascorbate analysis (see below). Plasma total cholesterol concentration was measured with a total cholesterol assay kit (Sigma). Residual plasma was pooled, argon-flushed, and stored at 4°C for ≤12 hours before size-exclusion chromatography and ex vivo oxidation with 2,2'-azo bis(2-amino-2-nitropropane) hydrochloride (5 mmol/L final concentration) as antioxidant. Such storage does not significantly alter plasma lipid oxidizability.

**Methods**

**Materials**

C18:2 and cholesteryl arachidonate (together called cholesteryl esters [CE]), nonesterified cholesterol (NEC), 5,6-cholen-3\B\B-19-diol (19-hydroxycholesterol), ascorbate, formalin, EDTA, glyceral, butylated hydroxytoluene and chloramphenicol were obtained from Sigma Chemical Co. 7KC was from Steraloids Inc (Wilton). VitE (RRR-\( \alpha \)-tocopherol) and CoQ\(_{10}\) were generous gifts from Henkel BioRad. DMSO was from Matheson, and calcium, magnesium, and chloride-free Dulbecco’s PBS (Sigma) was prepared from nanopure water and stored over chelating resin and kept frozen at \( -80°C \) for lipid and antioxidant analyses or diluted in 5% metaphosphoric acid (1:1 vol/vol) and stored at \(-80°C\) for ascorbate analysis (see below). Plasma total cholesterol concentration was measured with a total cholesterol assay kit (Sigma). Residual plasma was pooled, argon-flushed, and stored at 4°C for ≤12 hours before size-exclusion chromatography and ex vivo oxidation with 2,2'-azo bis(2-amino-2-nitropropane) hydrochloride (5 mmol/L final concentration) as antioxidant. Such storage does not significantly alter plasma lipid oxidizability.

**Aortic Sampling for Biochemical and Histologic Analyses**

Procedures were performed largely as described previously. Briefly, mice were perfused at near-physiological pressure with buffer A (PBS with 1 mmol/L EDTA and 20 \( \mu \)mol/L butylated hydroxytoluene). For biochemistry, aortas (control, \( n=12 \); VitE, CoQ\(_{10}\), and VitE + CoQ\(_{10}\); \( n=15 \) per group) were excised, and hearts and ascending and descending aortas past the femoral bifurcation were cleaned. Aortas then were randomly sorted into 3 separate groups of 4 to 6 and placed immediately in cold buffer B (buffer A containing 1 protease inhibitor tablet per 150 mL, 0.008% gentamicin, and 0.008% chloramphenicol). Aortas were then stored at \(-80°C\) until processing for biochemical analyses, excluding determination of F\(_2\)-isoprostanes and arachidonic acid. For histology, hearts and aortas of separate mice (control, \( n=14 \); VitE; CoQ\(_{10}\); and VitE + CoQ\(_{10}\); \( n=9 \) each) were excised. An upper portion of each aorta (to the third pair of intracostal arteries) was then placed in 4% vol/vol formaldehyde in saline overnight, before being transferred into 0.1% vol/vol formaldehyde in saline solution. The unfixed lower portion (from the fourth pair of intracostal arteries to the femoral bifurcation) was placed in buffer B and kept frozen at \(-80°C\) until analysis for F\(_2\)-isoprostanes and arachidonic acid (see below). Fixed tissue was transported to AstraZeneca for lesion assessment, performed in a blinded fashion by morphometry at the aortic root and arch and descending thoracic aorta as described previously in detail.
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For analysis of total cholesterol and 7KC, 10- and 100-μL aliquots, respectively, of the above isopropanol extracts were saponified after transfer to a screwcap tube and addition of diethyl ether (2.5 mL) and a methanolic solution of potassium hydroxide (20% wt/vol; 2.0 mL). 19-Hydroxycholesterol and cholesteryl propylether were analyzed after transfer to a screwcap tube and addition of diethyl ether (2.5 mL) and hexane (2.5 mL) were added. Extracts were then mixed vigorously (30 s) and centrifuged (1600g, 5 minutes, and 10°C). The ether/hexane phase was evaporated under vacuum and the extracts redissolved in heptane:isopropanol (95:5 vol/vol). 19-Hydroxycholesterol and cholesteryl propylether were measured routinely due to the unstable nature of CoQ10. CoQ9 and CoQ10 were measured routinely due to the unstable nature of CoQ10.39 CoQ9 and CoQ10 were measured routinely due to the unstable nature of CoQ10.39 CoQ9 and CoQ10 were measured routinely due to the unstable nature of CoQ10.39 CoQ9 and CoQ10 were measured routinely due to the unstable nature of CoQ10.39 CoQ9 and CoQ10 were measured routinely due to the unstable nature of CoQ10.39

TABLE 1. Concentrations of Plasma Lipids and Antioxidants After 24 Weeks of Intervention

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>VitE</th>
<th>CoQ10</th>
<th>VitE + CoQ10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol</td>
<td>22.7±6.3</td>
<td>22.5±5.1</td>
<td>22.8±4.4</td>
<td>22.7±4.2</td>
</tr>
<tr>
<td>NEC</td>
<td>9.3±2.0</td>
<td>9.9±2.4</td>
<td>10.0±3.3</td>
<td>11.0±2.6</td>
</tr>
<tr>
<td>C20:4</td>
<td>0.57±0.25</td>
<td>0.48±0.23</td>
<td>0.54±0.25</td>
<td>0.60±0.26</td>
</tr>
<tr>
<td>C18:2</td>
<td>3.4±1.1</td>
<td>3.9±1.3</td>
<td>3.8±1.2</td>
<td>4.0±1.1</td>
</tr>
<tr>
<td>LOOH</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>VitE</td>
<td>49.6±21.1</td>
<td>153±56.6</td>
<td>35.6±16.0</td>
<td>126±37.9</td>
</tr>
<tr>
<td>CoQ9</td>
<td>1.0±0.4</td>
<td>1.2±0.3</td>
<td>1.1±0.4</td>
<td>1.1±0.3</td>
</tr>
<tr>
<td>CoQ10</td>
<td>0.1±0.04</td>
<td>0.1±0.06</td>
<td>6.3±2.6</td>
<td>7.3±2.9</td>
</tr>
<tr>
<td>Total CoQ</td>
<td>1.1±0.4</td>
<td>1.3±0.4</td>
<td>7.4±2.9</td>
<td>8.4±2.8</td>
</tr>
<tr>
<td>Ascorbate</td>
<td>157±28</td>
<td>166±48</td>
<td>170±39</td>
<td>186±34</td>
</tr>
</tbody>
</table>

ApoE−/− mice were fed high-fat chow without (control) or with VitE, CoQ10, or VitE + CoQ10 for 24 weeks. After intervention, body weights were measured, plasma isolated, and concentrations of plasma lipids and antioxidants assessed. Values for total cholesterol, NEC, and CE are expressed in mmol/L, and values for antioxidants are expressed in μmol/L. Total CoQ represents the sum of CoQ9 and CoQ10. Data shown represent mean ± SD from 10 individual plasma samples from control or supplemented animals. Limit of detection of LOOH was 4 pmol.

*P<0.05 vs control; †P<0.05 vs CoQ10; ‡P<0.05 vs VitE.

Results

Plasma Lipids and Antioxidants

Effects of 24 weeks of supplementation of apoE−/− mice with VitE, CoQ10, or VitE + CoQ10 on plasma lipids and antioxidants are summarized in Table 1. None of the supplements significantly altered concentrations of total cholesterol, NEC, CE, and ascorbate. Supplementation with VitE or VitE + CoQ10 significantly increased plasma concentration of VitE =3-fold (P<0.001), whereas supplementation with CoQ10 or VitE + CoQ10 increased the concentration of total COQ =7-fold (P<0.001). This increase was solely due to an increase in CoQ10 (Table 1).

Plasma Lipoprotein Profile

Plasma from mice treated with VitE + CoQ10 contained increased levels VLDL compared with control mice, whereas supplementation with VitE or CoQ10 had a comparatively minor effect (Figure 1 and Table 2). LDL levels were decreased in mice treated with VitE, whereas HDL levels were similar in all groups (Table 2). Lipid analysis of the individual lipoprotein classes indicated that most of the increased plasma VitE and CoQ10, which resulted from supplementation with CoQ10 or VitE, respectively, was located in VLDL (Table 2).
Plasma Oxidizability

Plasma lipids from mice supplemented with CoQ10 or VitE were more resistant to peroxidation induced by peroxyl radicals compared with plasma from control or VitE-supplemented mice (Figure 2). As expected, $80\%$ of supplemented CoQ10 or endogenous CoQ9 was present as CoQ10 H2 and CoQ9 H2, respectively (not shown). Also, the time during which lipid peroxidation was effectively suppressed corresponded to the time required for the consumption of ascorbate and ubiquinols (not shown).

Tissue Concentrations of VitE and CoQ10

Supplementation with VitE or VitE+CoQ10 significantly increased VitE in all tissues examined, including aortas ($P<0.05$; Figure 3 and Table 3). In contrast, neither CoQ10 nor CoQ9 was increased significantly in heart, brain, or skeletal muscle after supplementation with CoQ10 or VitE+CoQ10 for 24 weeks (Figure 3). However, aortic content of CoQ10 increased >10-fold with CoQ10 and VitE+CoQ10 supplements ($P<0.001$), which resulted in a 2-fold increase in content of total CoQ ($P<0.02$; Figure 3 and Table 3). Between 20% and 50% of aortic CoQ was present as CoQ10 H2 or CoQ9 H2 (not shown), which indicates that the sample workup procedure used largely prevents inadvertent

**TABLE 2. Lipids and Antioxidants Present in Lipoprotein Classes Prepared From Plasma of ApoE−/− Mice After 24 Weeks of Intervention**

<table>
<thead>
<tr>
<th>Lipoprotein</th>
<th>Control</th>
<th>VitE</th>
<th>CoQ10</th>
<th>VitE+CoQ10</th>
</tr>
</thead>
<tbody>
<tr>
<td>VLDL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NEC</td>
<td>4.4</td>
<td>4.7</td>
<td>5.3</td>
<td>6.8</td>
</tr>
<tr>
<td>C20:4</td>
<td>120</td>
<td>155</td>
<td>164</td>
<td>413</td>
</tr>
<tr>
<td>C18:2</td>
<td>1508</td>
<td>1511</td>
<td>1486</td>
<td>2836</td>
</tr>
<tr>
<td>VitE</td>
<td>31</td>
<td>92.5</td>
<td>20.5</td>
<td>81.6</td>
</tr>
<tr>
<td>Total CoQ</td>
<td>0.46</td>
<td>0.36</td>
<td>2.0</td>
<td>4.0</td>
</tr>
<tr>
<td>LDL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NEC</td>
<td>2.1</td>
<td>1.2</td>
<td>1.7</td>
<td>2.2</td>
</tr>
<tr>
<td>C20:4</td>
<td>80</td>
<td>58</td>
<td>54</td>
<td>87</td>
</tr>
<tr>
<td>C18:2</td>
<td>603</td>
<td>328</td>
<td>341</td>
<td>409</td>
</tr>
<tr>
<td>VitE</td>
<td>13</td>
<td>15.2</td>
<td>4.9</td>
<td>13.5</td>
</tr>
<tr>
<td>Total CoQ</td>
<td>0.19</td>
<td>0.05</td>
<td>0.30</td>
<td>0.44</td>
</tr>
<tr>
<td>HDL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NEC</td>
<td>0.17</td>
<td>0.17</td>
<td>0.19</td>
<td>0.28</td>
</tr>
<tr>
<td>C20:4</td>
<td>16</td>
<td>16</td>
<td>11</td>
<td>23</td>
</tr>
<tr>
<td>C18:2</td>
<td>73</td>
<td>60</td>
<td>46</td>
<td>101</td>
</tr>
<tr>
<td>VitE</td>
<td>1.0</td>
<td>3.2</td>
<td>0.3</td>
<td>1.9</td>
</tr>
<tr>
<td>Total CoQ</td>
<td>0</td>
<td>0.05</td>
<td>0.05</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Plasma obtained from control or supplemented apoE−/− mice was pooled and subjected to size-exclusion chromatography. Two consecutive separations, each with 300 μL undiluted plasma, were performed for each group. Fractions from both injections and corresponding to VLDL, LDL, and HDL plus mouse serum albumin (MSA) (indicated in Figure 1) were collected, pooled, and analyzed for lipids and antioxidants. Data shown represents nmol of each analyte (except NEC, which is expressed in μmol) present in each class of lipoprotein derived from 600 μL of plasma pooled from 10 mice.
oxidation (ubiquinols are more sensitive to autoxidation than VitE). A consistent trend noted was that CoQ10 supplements decreased VitE concentrations in all tissues. Thus, extent of increase in VitE concentration in tissues was less in VitE+CoQ10 supplemented mice than in mice supplemented with VitE alone.

**Aortic Levels of Neutral and Oxidized Lipids**

Table 3 summarizes aortic concentration of lipids after 24 weeks of intervention. Compared with controls, aortas from all treated groups exhibited a significant decrease in levels of NEC and total cholesterol, with the order of efficacy VitE, CoQ10, and VitE+CoQ10 (Table 3). Such a decrease in aortic cholesterol content in the absence of a hypolipidemic effect (Table 1) is consistent with an antiatherogenic activity of the treatments. Aortic concentrations of C18:2 and cholesterol arachidonate, the 2 major oxidizable CE, were not altered content of F2-isoprostanes when expressed in absolute amount or per arachidonate (not shown).

To assess the effect of VitE and CoQ10 supplementation on aortic lipid oxidation, aortic concentrations of LOOH and 7KC were measured. Both types of oxidized lipids were detected in the aortas (Table 4). Identity of LOOH was verified by treating the organic extract with NaBH4. This resulted in disappearance of chemiluminescence-positive peaks coeluting with standards of LOOH and appearance of chemiluminescence-positive peaks coeluting with standards of ubiquinols (not shown). Treatment with VitE+CoQ10 significantly decreased both absolute concentration and the ratio of LOOH:CE (P<0.05; Table 4). Treatment with CoQ10 alone also decreased lipid-standardized levels of LOOH by ≈40%, although this did not reach statistical significance. In contrast, mean values of lipid-standardized LOOH were increased nonsignificantly by 25% in aortas from VitE-treated animals.

All treatments decreased absolute concentration of aortic 7KC, with a significant decrease apparent in mice treated with VitE or VitE+CoQ10 (P<0.05). However, these effects were no longer seen when aortic 7KC concentrations were standardized for total NEC content (Table 4). However, concentration of F2-isoprostanes determined varied greatly between different aortas within each treatment group and was 100-fold and 10-fold lower than LOOH and 7KC, respectively, when expressed per parent molecule (data not shown).

**Morphometry**

After 24 weeks of high-fat diet, lesions of grossly comparable morphology were found at all aortic sites examined (Figure 4), with necrotic cores containing cholesterol crystals observed frequently (not shown). ANOVA indicated that treatments significantly affected lesion size in a site-dependent manner, as indicated by a significant interaction term (P=0.001). Supplementation with VitE+CoQ10 significantly decreased lesion size at all sites examined (P≤0.05) (Figure

### Table 3. Aortic Lipids and Antioxidants After 24 Weeks of Intervention

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>VitE</th>
<th>CoQ10</th>
<th>VitE+CoQ10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol</td>
<td>875±128</td>
<td>468±66*</td>
<td>530±46*</td>
<td>426±81*</td>
</tr>
<tr>
<td>NEC</td>
<td>440±52</td>
<td>320±35*</td>
<td>354±12*</td>
<td>276±43*</td>
</tr>
<tr>
<td>C20:4</td>
<td>12.4±1.7</td>
<td>9.4±1.7</td>
<td>10.1±0.6</td>
<td>10.7±2.5</td>
</tr>
<tr>
<td>C18:2</td>
<td>30.2±5.2</td>
<td>29.2±3.9</td>
<td>25.4±5.5</td>
<td>29.3±4.9</td>
</tr>
<tr>
<td>VitE</td>
<td>1069±129</td>
<td>3309±892†</td>
<td>496±38*</td>
<td>2157±489††</td>
</tr>
<tr>
<td>CoQ9</td>
<td>140±26</td>
<td>128±20</td>
<td>127±1</td>
<td>126±23</td>
</tr>
<tr>
<td>CoQ10</td>
<td>17±2</td>
<td>14±3</td>
<td>199±38†</td>
<td>218±45‡</td>
</tr>
<tr>
<td>Total CoQ</td>
<td>157±28</td>
<td>142±23</td>
<td>326±39*</td>
<td>344±68*</td>
</tr>
<tr>
<td>Ascorbate</td>
<td>431±269</td>
<td>629±552</td>
<td>433±465</td>
<td>995±752</td>
</tr>
</tbody>
</table>

Aortas were obtained from control or supplemented apoE−/− mice, homogenized, and analyzed for parameters indicated. Data shown represent mean±SD from 3 groups of pooled aortas (n=4 to 5) from control or supplemented animals (control, n=12; VitE, CoQ10, and VitE+CoQ10, n=15 each). Values for total cholesterol, NEC, and CE are expressed in nmol/mg protein, and values for antioxidants are reported in pmol/mg protein. Total CoQ is the sum of CoQ9 and CoQ10.

### Table 4. Concentrations of Aortic Oxidized Lipids After 24 Weeks of Intervention

<table>
<thead>
<tr>
<th>Oxidized Lipid</th>
<th>Control</th>
<th>VitE</th>
<th>CoQ10</th>
<th>VitE+CoQ10</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOOH, pmol/mg protein</td>
<td>86±27</td>
<td>84.8±38</td>
<td>48±36</td>
<td>36±2*</td>
</tr>
<tr>
<td>LOOH/CE, mmol/mol</td>
<td>2.0±0.3</td>
<td>2.5±1.4</td>
<td>1.2±0.9</td>
<td>0.9±0.2*</td>
</tr>
<tr>
<td>7KC, nmol/mg protein</td>
<td>0.18±0.01</td>
<td>0.12±0.04*</td>
<td>0.14±0.02</td>
<td>0.08±0.02*</td>
</tr>
<tr>
<td>7KC/total Cholesterol, mmol/mol</td>
<td>0.21±0.02</td>
<td>0.25±0.07</td>
<td>0.27±0.06</td>
<td>0.18±0.03</td>
</tr>
</tbody>
</table>

Pooled aortas from control or supplemented apoE−/− mice (control, n=12; VitE, CoQ10, and VitE+CoQ10, n=15 each) were homogenized and analyzed for parameters indicated. Data shown for LOOH and 7KC represent mean±SD from 3 groups of pooled aortas from control or supplemented animals. CE represents C18:2 plus C20:4.
A previous study 27 showed that 0.2% VitE supplements decreased lesions in the aortic arch and thoracic aorta of apoE−/− mice by 60%. The aim of the present study was to test whether cosupplementation with CoQ10 further enhanced this antiatherogenic effect of VitE. Because 1% CoQ10 alone significantly decreases lesions in this animal model, 30 we compared the effect of 0.2% VitE with that of 0.5% CoQ10 and 0.2% VitE+0.5% CoQ10. We show that overall the antiatherogenic activity of VitE+CoQ10 was greater than that of VitE or CoQ10 supplementation alone. Inhibition of lesion formation was associated with a decrease in the aortic content of LOOH, a marker of primary lipoprotein lipid peroxidation. A further key finding was that VitE+CoQ10 increasingly inhibited disease along the aortic tree, similar to that reported recently for probucol along apoE−/− mice. 38 except that the intervention used in the present study reduced lesions also in the aortic root, whereas probucol enhances atherogenesis at that site. 38,45

How VitE+CoQ10 supplements inhibited atherosclerosis is unknown, although it significantly increased plasma and aortic concentrations of VitE and CoQ10 without lowering circulating concentration of cholesterol. This phenomenon indicates that a hypocholesteremic effect cannot explain the observed effect, in contrast to several previous animal interventions with antioxidants, including VitE, in which a confounding hypolipidemic effect was reported. 24 In consideration of the LDL oxidation theory, 1 antioxidants commonly are thought to attenuate atherosclerosis by inhibiting lipoprotein oxidation. Several lines of evidence suggest that such activity may have contributed to the antiatherosclerotic effect of VitE+CoQ10 supplements. Thus, VitE+CoQ10 treatment increased VitE and CoQ10H2 in plasma and the oxidation resistance of plasma lipids toward ex vivo peroxidation by aqueous peroxyl radicals, consistent with our previous study with human LDL. 33 This increased resistance of plasma lipids to peroxidation was associated with a significant decrease in content of aortic LOOH, independent of whether this oxidation parameter was expressed in absolute terms or lipid standardized. Together, these results support but do not prove the LDL oxidation theory.

Similar to VitE+CoQ10, supplementation with CoQ10 alone also inhibited ex vivo plasma lipid peroxidation and decreased aortic LOOH, although the latter did not reach significance. We observed recently that 1% wt/wt CoQ10 significantly decreased parent lipid-standardized LOOH in the vessel wall. 50 In the present study, the lower dose of CoQ10 used resulted in somewhat lower aortic concentrations of CoQ10, which may explain the lower efficacy found in the present study. In both studies, supplementation with CoQ10 was associated with inhibition of atherosclerosis of similar magnitude, except for aortic arch in the present study. Together, the findings support an antiatherogenic effect of CoQ10 in apoE−/− mice, albeit less pronounced than that seen with VitE+CoQ10.

Treatment with VitE alone failed to lower aortic LOOH, which indicates that VitE supplements alone may not inhibit primary lipid peroxidation despite the observed 3-fold increase in tissue content of the vitamin. In contrast, cosupplementation with VitE+CoQ10 significantly decreased aortic LOOH even though aortic VitE was increased only 2-fold. These results are consistent with, although not conclusive proof of, lipoprotein lipid peroxidation in the vessel wall proceeding through tocopherol-mediated peroxidation and indicate that coantioxidants inhibit lipoprotein oxidation in vivo. 24 The results also are consistent with the proposal 13 that cosupplementation of VitE with a lipid-soluble coantioxidant such as CoQ10H2 is a more effective antioxidant strategy than supplementation with VitE alone.

We also measured 7KC as an index of in vivo lipid oxidation. In contrast to LOOH, this parameter of secondary lipid oxidation, when expressed in a parent lipid-standardized manner, was not affected significantly by any of the treatments. In vitro experiments with LDL suggest that substantial accumulation of 7KC does not occur until after depletion of VitE. 46 However, our present (Table 3) and previous study 37 clearly show that VitE does not become depleted in the aortas of apoE−/− mice even after 6 months of high-fat diet, when
substantial lesions have formed. Thus, VitE supplements may not be expected to decrease aortic 7KC. This assumes that the measurement of 7KC reflects lipoprotein oxidation, although its accumulation in cells suggests that it may represent cellular rather than lipoprotein lipid oxidation. What is clear from the present study is that compared with LOOH, 7KC is a minor product of lipid oxidation in the vessel wall of apoE−/− mice, given that only 0.02% of total cholesterol was present as 7KC, whereas 0.2% of CE was detected as LOOH. This is consistent with the fact that compared with NEC, CE are chemically more susceptible to oxidation. We are not aware of a previous study that examined the effect of VitE (or CoQ10) supplement on oxysterols in apoE−/− mice. However, a recent study reported that feeding apoE−/− mice the isoflavon glabridin reduced both atherogenesis and the aortic levels of oxysterols including 7KC when standardized for wet weight of tissue. Similarly, in the present study, all supplements decreased aortic content of 7KC when expressed per aortic protein. However, attenuation of the extent of atherosclerosis by definition means attenuation of aortic cholesterol content as observed in the present study. Therefore, to distinguish an effect on disease burden (or lipid load) versus lipid oxidation within the vessel wall, it is necessary to standardize aortic content of oxysterols to cholesterol. When standardized, none of the supplements inhibited aortic content of 7KC.

Direct evidence for a causative link between inhibition of lipoprotein oxidation and atherosclerosis is scarce. Perhaps the most direct support for such a correlation is the observation that VitE reduced aortic content of isoprostane-F2α-VI and lesion size in apoE−/− mice. However, how precisely aortic isoprostane-F2α-VI relates to in vivo lipoprotein oxidation and/or atherogenesis is not known. For example, Pratico et al determined isoprostanes in hydrolyzed total lipid extracts of aortas, so that this measure is not specific for lipoproteins. Also, where examined, F2-isoprostanes in atherosclerotic lesions were reported to be associated primarily with foam cells. In contrast, the LOOH measured in the present study are found in lesion lipoproteins and are derived from the major oxidizable lipids associated with lipoproteins (ie, CE and triglycerides), so that this measure may reflect in vivo lipoprotein oxidation. Measuring accumulation of these LOOH in the vessel wall of LDL receptor-deficient rabbits, we recently observed that complete prevention of lipid peroxidation was not associated with inhibition of atherosclerosis. This finding suggests that lipoprotein lipid oxidation in the vessel wall can be dissociated from atherogenesis. Antioxidants such as CoQ10 and VitE may inhibit atherosclerosis by means other than inhibition of lipoprotein oxidation. For example, at the pharmacological dosage used in the present study, VitE can inhibit smooth muscle cell proliferation, platelet aggregation, and interleukin-1β release from monocytes in vitro. Also, VitE and CoQ10 may improve endothelial dysfunction in vivo.

In the present study, supplementation with 0.2% VitE alone had a moderate antiatherogenic effect in the aortic root only, whereas Pratico et al observed a ~60% decrease in aortic lesion area. Several differences between the 2 studies may explain the apparent discrepancy. We used a high-fat diet, which resulted in total plasma cholesterol of ~845 mg/dL (~22 mmol/L), whereas Pratico et al used a normal chow and reported plasma total cholesterol of ~500 mg/dL. Previous studies by others in hamsters suggest that the antiatherosclerotic activity of VitE is lost at plasma cholesterol concentrations >270 mg/dL. Thus, the comparatively higher cholesterol levels observed in the present study may have masked an antiatherogenic effect of VitE, although this requires further investigation. Shaish and coworkers recently reported that a combination of 0.05% VitE and 0.05% β-carotene was ineffective in preventing atherosclerosis in apoE−/− mice, consistent with both the moderate antiatherogenic activity of VitE observed in the present study and the overall disappointing results obtained with VitE supplements in animals and humans.

Extent of antiatherogenic activity observed in the present study with 0.5% CoQ10 is comparable to that observed recently with 1% CoQ10. This suggests that with the dosage used in the present study, we achieved an antiatherogenic effect near the maximum that can be achieved with this CoQ10 alone. This effect was obtained with a slightly smaller increase in aortic content of CoQ10 compared with that observed with a 1% supplementation. Therefore, 0.5% CoQ10 appears to be a suitable dose to test a beneficial effect of the coenzyme on the antiatherosclerotic activity of 0.2% VitE reported by Pratico et al. By showing that VitE+CoQ10 cosupplementation is more antiatherogenic than VitE or CoQ10 alone, the present study shows a benefit of the combination over the single antioxidant supplement.

We chose CoQ10 because it enriches lipoproteins with CoQ10:H2 that provides coantioxidation localized to where oxidation takes place. Interestingly, many antioxidants that inhibit atherosclerosis in animals, such as butylated hydroxytoluene, N,N′-diphenyl-phenylenediamine, and BO-653, are also lipid-soluble coantioxidants. Just as normal chow for laboratory animals is supplemented with VitE, addition of a coantioxidant alone may be seen as a form of cosupplementation with VitE plus coantioxidant. However, supplementation with a coantioxidant does not always attenuate atherosclerosis, although it prevents aortic lipid peroxidation.

Antiatherogenic efficacy of VitE+CoQ10 increased with increasing distance from the heart for presently unknown reasons, in a manner similar to what we described recently for probucol. However, even if this regional variability is considered, we cannot establish a clear link to any of the measures of oxidation used, and in the case of probucol, inhibition of atherosclerosis was observed without inhibition of aortic lipoprotein lipid per(oxidation). What is clear is that combining antioxidants with different properties increased overall antiatherogenic efficacy to an extent comparable to that of probucol in the aorta but also reduced lesion size in the aortic root.

A discrepancy seems to exist between efficacy by which the supplements decrease lesions versus aortic cholesterol. However, the difference in antiatherosclerotic effect between the combined treatment versus either antioxidant alone is most pronounced in the descending thoracic aorta (Figure 4). At that site, mean cross-sectional areas are only ~10% of that in the arch. Thus, in mass terms, the contribution of the descending thoracic aorta to lipid accumulation in the entire aorta (which we used for biochemistry) is limited.
In summary, the present study shows that supplementation of apoE−/− mice with VitE + CoQ10 is a more effective antiatherogenic treatment than supplementation with CoQ10 or VitE alone. This antiatherogenic activity is associated with a decrease in the aortic concentration of LOOH but not 7KC. Further studies are required to establish whether the antiatherogenic activity of VitE + CoQ10 reflects the ability of the antioxidants to inhibit lipoprotein oxidation in the vessel wall.

Acknowledgments

This work was supported by the Australian National Health and Medical Research grant 970098 to R.S. We thank Kaneka Corp and Henkel Corp for the generous gift of CoQ10 and VitE, respectively. We also thank J. Letters and P. Gabrielson for excellent technical assistance and the animal house staff at the Heart Research Institute for the maintenance of apoE−/− mice.

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Dietary Cosupplementation With Vitamin E and Coenzyme Q10 Inhibits Atherosclerosis in Apolipoprotein E Gene Knockout Mice
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doi: 10.1161/01.ATV.21.4.585

*Arteriosclerosis, Thrombosis, and Vascular Biology* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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