Dietary Cosupplementation With Vitamin E and Coenzyme Q_{10} 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 (CoQ_{10}, which is reduced during intestinal uptake to the antioxidant ubiquinol-10, $CoQ_{10}H_2$) 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 CoQ_{10} 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% CoQ_{10}, or 0.2% VitE plus 0.5% CoQ_{10} (VitE+CoQ_{10}) for 24 weeks. None of the supplements affected plasma cholesterol concentrations, whereas in the VitE and CoQ_{10} groups, plasma level of the respective supplement increased. Compared with control, plasma from CoQ_{10} or VitE+CoQ_{10} 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 CoQ_{10} supplementation increased CoQ_{10} only in plasma and aorta and lowered tissue VitE. All treatments significantly lowered aortic cholesterol compared with control, but only VitE+CoQ_{10} 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+CoQ_{10} 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. CoQ_{10} significantly inhibited atherosclerosis at aortic root and arch, whereas VitE decreased disease at aortic root only. Thus, in apoE−/− mice, VitE+CoQ_{10} supplements are more antiatherogenic than CoQ_{10} or VitE supplements alone and disease inhibition is associated with a decrease in aortic lipid hydroperoxides but not 7-ketocholesterol. **(*Arterioscler Thromb Vasc Biol.* 2001;21:585-593.)**

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 lipids[3,4] and proteins[5–8] are present in human lesions and lipoprotein-like particles isolated from them. For example, in advanced human atherosclerotic lesions, ≈5% of cholesteryl linoleate (C18:2), the major oxidizable lipid in LDL, is oxidized and present primarily as hydroperoxides and respective alcohols[9] and oxoderivatives.[10] In addition, atherosclerotic lesions also contain oxysterols[11] and F_2-isoprostanes,[12,13] prostaglandin-like and nonenzymatic lipid oxidation products of arachidonate.[14] However, these secondary lipid oxidation products are localized predominantly in foam cells[12,15] 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-3β-OL-7-one (7-ketocholesterol [7KC]) and F_2-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-epiprostaglandin $F_{2\alpha}$ 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

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From the Biochemistry (S.R.T., S.B.L., R.S.) and Cell Biology (A.J.B.) Groups, The Heart Research Institute, Camperdown, NSW, Australia; Cardiovascular Pharmacology (K.P.), AstraZeneca R&D, Mölndal, Sweden; and Department of Medicine (K.D.C., T.A.M.), University of Western Australia, Royal Perth Hospital, Perth, Western Australia.

Reprint requests to Dr Roland Stocker, The Heart Research Institute, 145 Missenden Rd, Camperdown NSW 2050, Australia. E-mail r.stocker@hri.org.au

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atherogenesis,[21] for reasons largely unknown. Also, in Watanabe hyperlipidemic rabbits, prevention of aortic lipid peroxidation itself is not sufficient for inhibition of atherosclerosis,[22] which suggests that antioxidants that attenuate atherosclerosis may do so by means of actions in addition to or independent of inhibition of lipoprotein oxidation.[23]

Plasma lipoproteins contain several endogenous antioxidants with α-tocopherol (vitamin E [VitE]) and ubiquinol-10 ($CoQ_{10}H_2$) that represent important modulators of lipid peroxidation.[24,25] 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.[26] 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.[27]

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 uptake.[28] Recently, an antiatherogenic effect of CoQ_{10} was reported for rabbits fed an undefined preoxidized diet,[29] and we observed that 1% wt/wt CoQ_{10} significantly reduced atherosclerosis in apoE−/− mice.[30] $CoQ_{10}H_2$ is an effective coantioxidant,[31–33] and coenrichment with VitE plus $CoQ_{10}H_2$ (VitE+$CoQ_{10}H_2$) effectively inhibits LDL lipid peroxidation, whereas VitE supplementation alone promotes this process under the mild in vitro oxidizing conditions used.[33] We therefore proposed[33] that cosupplementation with VitE plus CoQ_{10} (VitE+CoQ_{10}) may be more antiatherogenic than either VitE or CoQ_{10} supplementation alone. In the present study, we show supporting evidence for this proposal and report differences with respect to the extent to which cosupplementation inhibits accumulation of different types of oxidized lipids within the vessel wall.

Methods

Materials

C18:2 and cholesteryl arachidonate (together called cholesteryl esters [CE]), nonesterified cholesterol (NEC), 5-cholesten-3β,19-diol (19-hydroxycholesterol), ascorbate, formalin, EDTA, glycerol, butylated hydroxytoluene and chloramphenicol were obtained from Sigma Chemical Co. 7KC was from Steraloids Inc (Wilton). VitE (*RRR*-α-tocopherol) and CoQ_{10} were generous gifts from Henkel Corp and Kaneka Corp, respectively. α-Tocotrienol was purified as described.[34] Authentic C18:2-OOH, used as a standard for LOOH, was prepared as described.[35] Authentic $CoQ_{10}H_2$, prepared as described,[36] was used while fresh as a standard for CoQ_9H_2 and $CoQ_{10}H_2$. Protease inhibitor cocktail tablets were from Boehringer, and gentamicin and chloramphenicol from Gibco BRL. Calcium- and magnesium chloride–free Dulbecco's PBS (Sigma) was prepared from nanopure water and stored over chelating resin (Chelex 100; BioRad) at 4°C for ≥24 hours to remove contaminating transition metals. All buffers were filtered and argon-flushed immediately before use. Organic solvents and all other chemicals used were of the highest quality available.

ApoE−/− Mice

Male C57BL/6J mice, homozygous for the disrupted apoE gene (apoE−/−) were purchased from Jackson Laboratories (Bar Harbor, Me), bred at The Heart Research Institute (Sydney, Australia), and fed standard mouse chow (Laboratory-Feed) until the age of 8 to 10 weeks.[37] Animals were then fed for 24 weeks ad libitum a high-fat diet containing 21.2 wt/wt fat and 0.15% wt/wt cholesterol without (controls; n=26) or with VitE (0.2% wt/wt; n=25), CoQ_{10} (0.5% wt/wt; n=24), or VitE+CoQ_{10} (CoQ_{10} 0.5% wt/wt, VitE 0.2% wt/wt; n=24). Diets were prepared by MJ Hoxey and Associates according to specifications of the Harlan Teklad diet TD88137, packaged and sealed under N_2, and stored at 4°C until use.

Plasma Analyses and Ex Vivo Oxidation

Plasma was prepared from blood as described.[37] Aliquots (50 μL) of the resulting plasma were extracted immediately into hexane and methanol[35] and stored 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-amidinopropane) hydrochloride (5 mmol/L final concentration) as oxidant.[37] Such storage does not significantly alter plasma lipid oxidizability.[33]

Aortic Sampling for Biochemical and Histologic Analyses

Procedures were performed largely as described previously.[37,38] Briefly, mice were perfused at near-physiological pressure with buffer A (PBS with 1 mmol/L EDTA and 20 μmol/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) were excised. The 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.[38]

Aortic Biochemistry

Pooled aortas were pulverized in liquid N_2, resuspended in 1.5 mL of buffer A, and homogenized as described.[37] A 50-μL aliquot of homogenate was added to an equal volume of 5% metaphosphoric acid for ascorbate analysis and a further 50 μL removed for protein determination with the bicinchoninic acid assay kit (Sigma). Remaining homogenate was extracted in 500-μL 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 μL of isopropanol.[35] This extract was analyzed for lipid-soluble antioxidants, NEC, CE, and LOOH by high-performance liquid chromatography (HPLC) as described.[35,39] LOOH were measured as a marker of primary lipoprotein lipid peroxidation because it is the primary and major lipid oxidation product formed in lipoproteins from apoE−/− mice undergoing oxidation.[40] A previous study has shown that 70% of [^{3}H]-C18:2-OOH added to mouse aorta before pulverizing is recovered as the hydroperoxide and 30% recovered as the corresponding alcohol and that [^{3}H]-C18:2 added to aortas before workup is not converted to [^{3}H]-C18:2-OOH or [^{3}H]-C18:2-OH.[37] To confirm the presence of LOOH, postcolumn chemiluminescence detection was used before and after borohydride treatment of samples.[41] All compounds detected were quantified by peak area comparison with authentic standards run under identical conditions.

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

Parameter	Control	VitE	CoQ_{10}	VitE+CoQ_{10}
Total cholesterol	22.7±6.3	22.5±5.1	22.8±4.4	22.7±4.2
NEC	9.3±2.0	9.9±2.4	10.0±3.3	11.0±2.6
C20:4	0.57±0.25	0.48±0.23	0.54±0.25	0.60±0.26
C18:2	3.4±1.1	3.9±1.3	3.8±1.2	4.0±1.1
LOOH	0	0	0	0
VitE	49.6±21.1	153±56.6*†	35.6±16.0	126±37.9*†
CoQ_9	1.0±0.4	1.2±0.3	1.1±0.4	1.1±0.3
CoQ_{10}	0.1±0.04	0.1±0.06	6.3±2.6*‡	7.3±2.9*‡
Total CoQ	1.1±0.4	1.3±0.4	7.4±2.9*‡	8.4±2.8*‡
Ascorbate	157±28	166±48	170±39	186±34

ApoE−/− mice were fed high-fat chow without (control) or with VitE, CoQ_{10}, or VitE+CoQ_{10} 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 CoQ_9 and CoQ_{10}. 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 CoQ_{10}; ‡$P<0.05$ vs VitE.

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 (both 100 μL of a 50-μg/mL solution in heptane/isopropanol 95:5 vol/vol) were added as internal standards for 7KC and total NEC, respectively. Tubes were flushed with argon and mixed overnight at 4°C before H_2O (2.0 mL) and hexane (2.5 mL) were added. Extracts were then mixed vigorously (30 s) and centrifuged (1600*g*, 5 minutes, and 10°C). The ether/hexane phase was evaporated under vacuum and the extracts redissolved in heptane:isopropanol (95:5 vol/vol) or isopropanol:acetonitrile:water (54:44:2 vol/vol/vol) for 7KC or total cholesterol HPLC determination, respectively.[16,42] For total cholesterol, a silica column (0.46×15 cm, 100 Å, 5 μm; Alltech) with guard column (3-μm particle size) was used with hexane:isopropanol:acetonitrile (94.8:4.6:0.6 vol/vol/vol) as the eluant at 1.0 mL/min monitored at 234 nm. Typical retention times of 7KC and 19-hydroxycholesterol were 14.8 and 28.3 minutes, respectively.

For analysis of F_2-isoprostanes, the pieces of thawed aortas (≈20 mg wet wt) were blotted dry, weighed, and homogenized in ice-cold chloroform:methanol (2:1 vol/vol). [D_4]-8-iso-prostaglandin $F_{2\alpha}$ (Cayman Chemicals) was added as an internal standard. After removal of an aliquot for arachidonate analysis (see below), the organic phase was dried under nitrogen and hydrolyzed by addition of 15% KOH and incubated for 1 hour at 45°C. F_2-isoprostanes were analyzed after solid-phase extraction and HPLC purification by electron-capture negative-ionization gas chromatography-mass spectrometry as described in detail.[43] For arachidonate analysis, phospholipids were separated from total lipid extracts by thin-layer chromatography on silica gel 60 F254-precoated aluminum sheets (Merck) with hexane:diethyl ether:acetic acid:methanol (170:40:4:4 vol/vol) as the solvent. Fatty acid methyl esters were prepared by treatment with 4% H_2SO_4 in methanol at 90°C for 20 minutes and analyzed by gas-liquid chromatography.[43] Peaks were identified by comparison with known standards. Arachidonate was quantified with heptadecanoic acid as the internal standard.

Analysis of VitE and CoQ_{10} Levels in Heart, Brain, and Skeletal Muscle

Concentrations of VitE, CoQ_{10}, and ubiquinone-9 (CoQ_9) were also determined in heart, brain, and hind-limb skeletal muscle. Whole tissues were homogenized in 2 mL of ice-cold buffer A, and a 50-μL aliquot was removed for protein determination. The remaining homogenate (200 μL) was extracted in 2 mL methanol and 10 mL hexane; the organic phase was resuspended in 200 μL of isopropanol and analyzed for VitE, CoQ_9, and CoQ_{10} by HPLC.[39] CoQ_{10} and CoQ_9 were measured routinely due to the unstable nature of $CoQ_{10}H_2$ and CoQ_9H_2.[44]

Statistics

Data on lesion size are presented as mean±SEM, and effects of supplements analyzed by 2-way ANOVA (SAS software) by use of log-transformed data, with supplements and aortic sites as factors. Because a significant interaction term existed between supplement and aortic site, treatment effect at each site measured was evaluated by Student's *t* test with log-transformed data. Biochemical parameters were compared by use of 1-way ANOVA or Student's *t* test (Systat 8.0 software; SPSS). Statistical significance was accepted at $P<0.05$.

Results

Plasma Lipids and Antioxidants

Effects of 24 weeks of supplementation of apoE−/− mice with VitE, CoQ_{10}, or VitE+CoQ_{10} 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+CoQ_{10} significantly increased plasma concentration of VitE ≈3-fold ($P<0.001$), whereas supplementation with CoQ_{10} or VitE+CoQ_{10} increased the concentration of total CoQ ≈7-fold ($P<0.001$). This increase was solely due to an increase in CoQ_{10} (Table 1).

Plasma Lipoprotein Profile

Plasma from mice treated with VitE+CoQ_{10} contained increased levels VLDL compared with control mice, whereas supplementation with VitE or CoQ_{10} 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 CoQ_{10}, which resulted from supplementation with CoQ_{10} or VitE, respectively, was located in VLDL (Table 2).

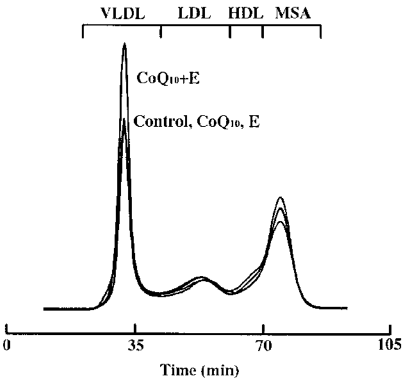

Figure 1. Plasma lipoprotein profile of apoE−/− mice fed a high-fat diet nonsupplemented or supplemented with VitE, CoQ_{10}, and VitE+CoQ_{10} for 24 weeks. After intervention, plasma was collected from individual mice (n=8 to 10 per group), pooled, diluted 1:10 with FPLC mobile phase. Then, 300 μL was subjected to size-exclusion chromatography and lipoprotein profile monitored at 280 nm as described previously.[37] Chromatograms shown are representative of 2 analyses of separate pools of plasma samples. Corresponding fractions collected for each lipoprotein class are indicated.

Plasma Oxidizability

Plasma lipids from mice supplemented with CoQ_{10} or VitE+CoQ_{10} 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 CoQ_{10} or endogenous CoQ_9 was present as $CoQ_{10}H_2$ and CoQ_9H_2, respectively (not shown).[30] Also, the time during which lipid peroxidation was effectively suppressed corresponded to the time required for the consumption of ascorbate and ubiquinols (not shown).

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

Lipoprotein	Control	VitE	CoQ_{10}	VitE+CoQ_{10}
VLDL				
NEC	4.4	4.7	5.3	6.8
C20:4	120	155	164	413
C18:2	1508	1511	1486	2836
VitE	31	92.5	20.5	81.6
Total CoQ	0.46	0.36	2.0	4.0
LDL				
NEC	2.1	1.2	1.7	2.2
C20:4	80	58	54	87
C18:2	603	328	341	409
VitE	13	15.2	4.9	13.5
Total CoQ	0.19	0.05	0.30	0.44
HDL				
NEC	0.17	0.17	0.19	0.28
C20:4	16	16	11	23
C18:2	73	60	46	101
VitE	1.0	3.2	0.3	1.9
Total CoQ	0	0	0.05	0.1

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.

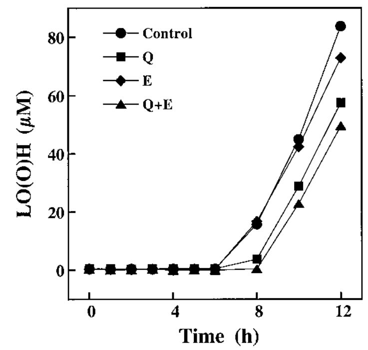

Figure 2. Dietary supplementation of apoE−/− mice with CoQ_{10} or VitE+CoQ_{10} but not VitE enhances oxidation resistance of plasma lipids exposed to aqueous peroxyl radicals. Pooled plasma prepared from blood of control (●), CoQ_{10} (■), VitE (◆) and VitE+CoQ_{10} (▲) mice (n=8 to 10 for each group) was exposed to 5 mmol/L AAPH and incubated in air at 37°C. At times indicated, aliquots of reaction mixture were removed and analyzed for LOOH. Table 1 gives initial concentrations of plasma parameters. Data shown are mean values from 1 oxidation experiment performed in duplicate with pooled plasma.

Tissue Concentrations of VitE and CoQ_{10}

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 CoQ_{10} nor CoQ_9 was increased significantly in heart, brain, or skeletal muscle after supplementation with CoQ_{10} or VitE+CoQ_{10} for 24 weeks (Figure 3). However, aortic content of CoQ_{10} increased >10-fold with CoQ_{10} and VitE+CoQ_{10} 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 $CoQ_{10}H_2$ or CoQ_9H_2 (not shown), which indicates that the sample workup procedure used largely prevents inadvertent

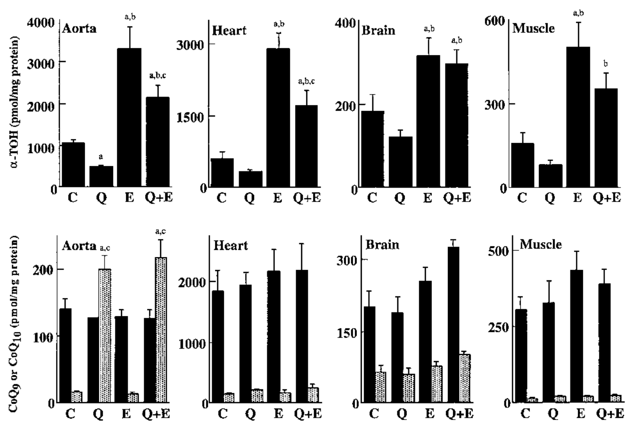

Figure 3. Tissue levels of VitE, CoQ_{10}, and CoQ_9 after dietary supplementation with VitE, CoQ_{10}, or VitE+CoQ_{10}. After 24 weeks' intervention, aorta (3 pools of 4 to 5 aortas), heart, brain, and skeletal muscle (n=5 for each group and tissue) were removed from control and supplemented animals, homogenized, and analyzed for VitE and CoQ_9 (■) and CoQ_{10} (□). Results are mean±SEM. [a]$P<0.05$ vs control; [b]$P<0.05$ vs CoQ_{10}; [c]$P<0.05$ vs VitE.

TABLE 3. Aortic Lipids and Antioxidants After 24 Weeks of Intervention

Parameter	Control	VitE	CoQ_{10}	VitE+CoQ_{10}
Total cholesterol	875±128	468±66*	530±46*	426±81*
NEC	440±52	320±35*	354±12*	276±43*
C20:4	12.4±1.7	9.4±1.7	10.1±0.6	10.7±2.5
C18:2	30.2±5.2	29.2±3.9	25.4±5.5	29.3±4.9
VitE	1069±129	3309±892*†	496±38*	2157±489*†‡
CoQ_9	140±26	128±20	127±1	126±23
CoQ_{10}	17±2	14±3	199±38*‡	218±45*‡
Total CoQ	157±28	142±23	326±39*	344±68*
Ascorbate	431±269	629±552	433±465	995±752

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, CoQ_{10}, and VitE+CoQ_{10}, 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 CoQ_9 and CoQ_{10}.

**P*<0.05 vs control; †*P*<0.05 vs CoQ_{10}; ‡*P*<0.05 vs VitE.

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+CoQ_{10} 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+CoQ_{10}>VitE>CoQ_{10} (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 cholesteryl arachidonate, the 2 major oxidizable CE, were not decreased significantly by any treatment.

To assess the effect of VitE and CoQ_{10} 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 $NaBH_4$.[41] 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+CoQ_{10} significantly decreased both absolute concentration and the ratio of LOOH:CE (*P*<0.05; Table 4). Treatment with CoQ_{10} 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 F_2-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). Compared with controls, none of the treatments significantly altered content of F_2-isoprostanes when expressed in absolute amount or per arachidonate (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+CoQ_{10} significantly decreased lesion size at all sites examined (*P*≤0.05) (Figure

TABLE 4. Concentrations of Aortic Oxidized Lipids After 24 Weeks of Intervention

Oxidized Lipid	Control	VitE	CoQ_{10}	VitE+CoQ_{10}
LOOH, pmol/mg protein	86±27	84.8±38	48±36	36±2*
LOOH/CE, mmol/mol	2.0±0.3	2.5±1.4	1.2±0.9	0.9±0.2*
7KC, nmol/mg protein	0.18±0.01	0.12±0.04*	0.14±0.02	0.08±0.02*
7KC/total Cholesterol, mmol/mol	0.21±0.02	0.25±0.07	0.27±0.06	0.18±0.03

Pooled aortas from control or supplemented apoE−/− mice (control, n=12; VitE, CoQ_{10}, and VitE+CoQ_{10}, 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.

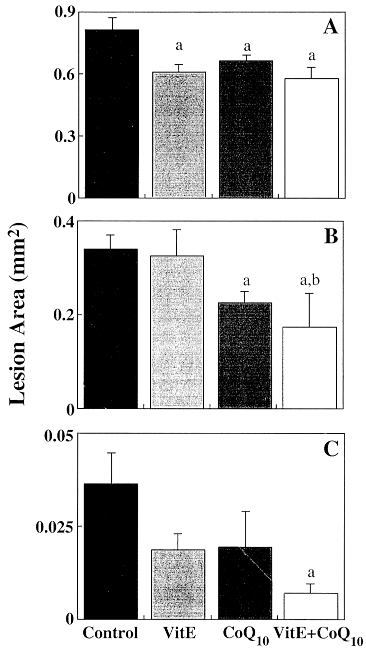

Figure 4. Dietary supplementation with VitE+CoQ_{10} for 24 weeks inhibits atherosclerosis in apoE−/− mice fed a high-fat diet. Atherosclerosis was assessed by morphometry at aortic root (A), aortic arch (B), and descending thoracic aorta (C). Data shown represent mean±SEM of 14, 9, 9, and 9 areas of control, VitE-, CoQ_{10}-, and VitE+CoQ_{10}-treated animals, respectively, for cross sections taken at aortic root, aortic arch, and descending thoracic aorta. $^{a}P<0.05$ vs control; $^{b}P<0.05$ vs VitE.

4). Extent of disease inhibition increased from the aortic root to the aortic arch and descending thoracic aorta, with ≈30%, 50%, and 80% inhibition, respectively, compared with respective sites in control animals. Supplementation with CoQ_{10} alone significantly decreased the size of lesions in the aortic root and arch ($P\leq0.05$) but not in descending thoracic aorta. VitE supplementation significantly decreased lesion size only in the aortic root ($P\leq0.05$). No significant differences were observed in size of lesions among any of the treatment groups except that animals supplemented with VitE+CoQ_{10} exhibited significantly smaller lesions in the aortic arch compared with VitE-supplemented mice ($P\leq0.05$).

Discussion

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 CoQ_{10} further enhanced this antiatherogenic effect of VitE. Because 1% CoQ_{10} alone significantly decreases lesions in this animal model,[30] we compared the effect of 0.2% VitE with that of 0.5% CoQ_{10} and 0.2% VitE+0.5% CoQ_{10}. We show that overall the antiatherogenic activity of VitE+CoQ_{10} was greater than that of VitE or CoQ_{10} 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+CoQ_{10} increasingly inhibited disease along the aortic tree, similar to that reported recently for probucol in 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+CoQ_{10} supplements inhibited atherosclerosis is unknown, although it significantly increased plasma and aortic concentrations of VitE and CoQ_{10} without lowering circulating concentration of cholesterol. This phenomenon indicates that a hypocholesterolemic 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+CoQ_{10} supplements. Thus, VitE+CoQ_{10} treatment increased VitE and $CoQ_{10}H_2$ 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+CoQ_{10}, supplementation with CoQ_{10} 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 CoQ_{10} significantly decreased parent lipid-standardized LOOH in the vessel wall.[30] In the present study, the lower dose of CoQ_{10} used resulted in somewhat lower aortic concentrations of CoQ_{10}, which may explain the lower efficacy found in the present study. In both studies, supplementation with CoQ_{10} 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 CoQ_{10} in apoE−/− mice, albeit less pronounced than that seen with VitE+CoQ_{10}.

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+CoQ_{10} 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[33] that cosupplementation of VitE with a lipid-soluble coantioxidant such as $CoQ_{10}H_2$ 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[15] 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 CoQ_{10}) supplement on oxysterols in apoE−/− mice. However, a recent study reported that feeding apoE−/− mice the isoflavan glabridin reduced both atherogenesis and the aortic levels of oxysterols including 7KC when standardized for wet weight of tissue.[47] 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-$F_{2\alpha}$-VI and lesion size in apoE−/− mice.[27] However, how precisely aortic isoprostane-$F_{2\alpha}$-VI relates to in vivo lipoprotein oxidation and/or atherogenesis is not known. For example, Pratico et al[27] determined isoprostanes in hydrolyzed total lipid extracts of aortas, so that this measure is not specific for lipoproteins. Also, where examined, F_2-isoprostanes in atherosclerotic lesions were reported to be associated primarily with foam cells.[12] In contrast, the LOOH measured in the present study are found in lesion lipoproteins[9] 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.[22] This finding suggests that lipoprotein lipid oxidation in the vessel wall can be dissociated from atherogenesis. Antioxidants such as $CoQ_{10}H_2$ and VitE may inhibit atherosclerosis by means other than inhibition of lipoprotein oxidation.[23] For example, at the pharmacological dosage used in the present study, VitE can inhibit smooth muscle cell proliferation,[48] platelet aggregation[49], and interleukin-1β release from monocytes in vitro.[50] Also, VitE[51] and CoQ_{10}[52] 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[27] 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.[27] Previous studies by others in hamsters suggest that the antiatherosclerotic activity of VitE is lost at plasma cholesterol concentrations >270 mg/dL.[53] 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[54] 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[24,26] and humans.[55]

Extent of antiatherogenic activity observed in the present study with 0.5% CoQ_{10} is comparable to that observed recently with 1% CoQ_{10}.[30] 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 CoQ_{10} alone. This effect was obtained with a slightly smaller increase in aortic content of CoQ_{10} compared with that observed with a 1% supplementation.[30] Therefore, 0.5% CoQ_{10} 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.[12] By showing that VitE+CoQ_{10} cosupplementation is more antiatherogenic than VitE or CoQ_{10} supplement alone, the present study shows a benefit of the combination over the single antioxidant supplement.

We chose CoQ_{10} because it enriches lipoproteins with $CoQ_{10}H_2$ that provides coantioxidation localized to where oxidation takes place. Interestingly, many antioxidants that inhibit atherosclerosis in animals, such as butylated hydroxytoluene,[56] *N*,*N'*-diphenyl-phenylenediamine,[57] and BO-653,[58] are also lipid-soluble coantioxidants.[59] Just as normal chow for laboratory animals is supplemented with VitE,[60] 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,[22] although it prevents aortic lipid peroxidation.

Antiatherogenic efficacy of VitE+CoQ_{10} increased with increasing distance from the heart for presently unknown reasons, in a manner similar to what we described recently for probucol.[38] 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.[38] 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.[38]

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+CoQ_{10} is a more effective antiatherogenic treatment than supplementation with CoQ_{10} 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+CoQ_{10} reflects the ability of the antioxidants to inhibit lipoprotein oxidation in the vessel wall.

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Dietary Cosupplementation With Vitamin E and Coenzyme Q_{10} Inhibits Atherosclerosis in Apolipoprotein E Gene Knockout Mice

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