Combined Vitamin C and Vitamin E Deficiency Worsens Early Atherosclerosis in Apolipoprotein E–Deficient Mice

Vladimir R. Babaev, Liying Li, Sanket Shah, Sergio Fazio, MacRae F. Linton, James M. May

Objective—To assess the role of combined deficiencies of vitamins C and E on the earliest stages of atherosclerosis (an inflammatory condition associated with oxidative stress), 4 combinations of vitamin supplementation (low C/low E, low C/high E, high C/low E, and high C/high E) were studied in atherosclerosis-prone apolipoprotein E–deficient mice also unable to synthesize their own vitamin C (gulonolactone oxidase−/−); and to evaluate the effect of a more severe depletion of vitamin C alone in a second experiment using gulonolactone oxidase−/− mice carrying the hemizygous deletion of SVCT2 (the vitamin C transporter).

Methods and Results—After 8 weeks of a high-fat diet (16% lard and 0.2% cholesterol), atherosclerosis developed in the aortic sinus areas of mice in all diet groups. Each vitamin-deficient diet significantly decreased liver and brain contents of the corresponding vitamin. Combined deficiency of both vitamins increased lipid peroxidation, doubled plaque size, and increased plaque macrophage content by 2- to 3-fold in male mice, although only plaque macrophage content was increased in female mice. A more severe deficiency of vitamin C in gulonolactone oxidase−/− mice with defective cellular uptake of vitamin C increased both oxidative stress and atherosclerosis in apolipoprotein E−/− mice compared with littersmates receiving a diet replete in vitamin C, again most clearly in males.

Conclusion—Combined deficiencies of vitamins E and C are required to worsen early atherosclerosis in an apolipoprotein E–deficient mouse model. However, a more severe cellular deficiency of vitamin C alone promotes atherosclerosis when vitamin E is replete. (Arterioscler Thromb Vasc Biol. 2010;30:1751-1757.)

Key Words: antioxidants ■ atherosclerosis ■ genetically altered mice ■ nutrition

Atherosclerosis is an inflammatory disease that is initiated, at least in part, by the interaction of oxidized low-density lipoprotein with cells of the vascular wall.1 A potential role for oxidative stress in the disease has led to the notion that it might be prevented by antioxidant treatment and especially by antioxidant vitamins, such as ascorbic acid (vitamin C) and α-tocopherol (vitamin E). However, recent large clinical trials2-4 have generally not supported the notion that these antioxidant vitamins can slow the progression of established atherosclerosis. Whether antioxidant vitamins can prevent or slow early atherosclerosis may be a more relevant question that is not readily tested in humans, given the prolonged asymptomatic period of the disease. Studies in animal models of atherosclerosis might provide support for such clinical trials, but these are also hampered by the fact that most mammals can synthesize vitamin C, making it difficult to deplete the vitamin. One exception is the guinea pig, which, like humans, lacks the enzyme gulonolactone oxidase (gulo) and, thus, the ability to synthesize vitamin C. Indeed, guinea pigs with early scurvy were shown more than 5 decades ago to develop lesions that, on histology, were indistinguishable from atherosclerosis in regions of large arteries exposed to hemodynamic stress.5 These lesions were reversed by provision of ascorbate in the diet.6 These findings were extended by Maeda et al7 using a mouse model in which gulo was deleted by targeted gene disruption. When placed on a diet deficient in vitamin C, the gulo−/− mice developed early scurvy after 4 to 5 weeks, and aortas showed disruption of the elastic lamina. However, no intimal lesions characteristic of atherosclerosis were observed. To investigate this issue in more detail, Nakata and Maeda8 crossed the gulo−/− mice with mice deficient in apolipoprotein E (apoE). The mice were fed normal chow diets (practically ascorbate free) and received supplementation with ascorbate in their drinking water with 2 levels of the vitamin: one that produces supranormal plasma and liver ascorbate concentrations, and the other that results in levels 25% to 30% of normal (still adequate to sustain normal weight gain and prevent scurvy). After 4 months of these vitamin C intakes, the mice had developed lipid-laden atherosclerotic plaques in the aortic sinus that increased in size after 9 months of continued supplementation. However, ascorbate intake did not affect lesion size at either time in either sex. Feeding a high-fat diet for 4 months increased plaque size, but there was still no difference in lesion size between the 2 ascorbate diets. On the other hand, compared with mice receiving the high ascorbate
supplements, mice receiving the low ascorbate supplements had plaques with 30% to 50% decreases in collagen content, larger necrotic cores, and decreased fibrosis. Although these results suggest a role for ascorbate in plaque morphology and possibly plaque stability, they fail to support the notion that marginal ascorbate deficiency affects atherosclerotic plaque development, size, or progression.

Despite the negative results with moderate vitamin C deficiency alone, several caveats are worth considering. First, there may be different effects of vitamin C deficiency at earlier points than 4 months because vitamin C is best known to affect early changes in atherosclerosis, such as endothelial dysfunction, and because irreversible changes may not have occurred at this early point. Second, there may be a cooperative effect of vitamins C and E in protecting arterial wall integrity, and a more severe deficiency of vitamin C may be required in the mouse model. Indeed, because lipid hydroperoxides in oxidized low-density lipoprotein appear to be required for lesion formation, the lipid-soluble vitamin E (α-tocopherol) might be better at quenching lipid hydroperoxide propagation compared with the water-soluble ascorbate. Moreover, ascorbate is capable of recycling α-tocopherol in lipid micelles and of sparing α-tocopherol in intact cells. Furthermore, 2 studies have shown that a severe deficiency of vitamin E as the result of lack of the α-tocopherol transfer protein increased atherosclerosis in apoE−/− mice. Third, a more significant depletion of vitamin C may be required to provide proof-of-principle evidence that supplementation has resulted in normal plasma and liver ascorbate. When deionized water was used (data not shown). This level of ascorbate supplementation has resulted in normal plasma and liver levels of vitamin C. All protocols were approved by the Animal Care and Use Committee of Vanderbilt University, Nashville, Tenn.

Diet and Supplements of Vitamins C and E

After weaning, male and female pups were maintained on normal chow and drinking water that contained 0.33 g/L ascorbate and 0.01 mmol/L ethylenediaminetetraacetic acid (EDTA), until the age of 5 weeks. All mice were then given high-fat diets (Harlan-Teklad, Madison, Wis). For experiment 1, the diets lacked vitamin C and either contained vitamin E (TD.07311, 135 IU/kg of vitamin E as DL-α-tocopheryl acetate, 16% vitamin E–stripped lard, and 0.2% cholesterol) or lacked vitamin E (TD.07310, approximately 5 IU/kg vitamin E, 16% vitamin E–stripped lard, and 0.2% cholesterol). For experiment 2, gulo−/−/apoE−/−/SVCT2−/− mice received a high-fat diet with vitamin E supplements, as noted for experiment 1.

For experiment 1, mice were randomly assigned to 1 of 4 groups with different levels of vitamin C and E supplementation. The groups were as follows (based on average individual consumption of 4 g/d feed and 5 mL/d water): high E/high C, 0.27 mg/d (0.6 IU/d) vitamin E plus 1.7 mg/d vitamin C; high E/low C, 0.27 mg/d vitamin E plus 0.17 mg/d vitamin C; low E/high C, approximately 0.01 mg/d vitamin E plus 1.7 mg/d vitamin C; and low E/low C, approximately 0.01 mg/d vitamin E plus 0.17 mg/d vitamin C. Vitamin E was supplemented by the supplier in the diet, whereas vitamin C was supplemented in the drinking water that also contained 0.01 mmol/L EDTA. The concentrations of vitamin C in the drinking water were as follows: high C, 0.33 g/L; and low C, 0.033 g/L.

For experiment 2, gulo−/−/apoE−/−/SVCT2−/− mice were randomly assigned to the same high or low content of vitamin C in the drinking water, as noted for experiment 1: high C, 0.33 g/L; and low C, 0.033 g/L.

Plasma Lipid Analyses and Assays of Ascorbate, α-Tocopherol, and Lipid Peroxidation Products

Plasma cholesterol and triglycerides were determined in mice that fasted for 4 hours, as previously described. For assays of ascorbate, weighed tissues (50 to 100 mg of wet weight) were homogenized in microfuge tubes using a plastic pestle in 0.1 mL of 25% metaphosphoric acid, wt/vol, followed by further homogenization in 0.35 mL of a buffer containing 0.1 mol/L Na₂HPO₄ and 0.05 mmol/L EDTA pH 8.0. For assay of ascorbate in serum, 0.1 mL of serum was treated with 400 mmol/L sodium perchlorate and mixed. The treated serum or tissue homogenates were centrifuged at 3°C for 1 minute at 13 000 g. Duplicate aliquots of supernatant were taken for assay of ascorbate. Vitamin E in tissues and plasma was assayed as α-tocopherol by high-performance liquid chromatography with electrochemical detection by the method of Lang et al. Malondialdehyde was measured in the heart using the high-performance liquid chromatographic method of Tebbe et al, as previously modified. F₂-isoprostanes were analyzed after Folch extraction, base hydrolysis, and derivatization, as previously described. in the Eicosanoid Core Laboratory at Vanderbilt University. In both assays, butylated hydroxytoluene was added during extraction and sample preparation to prevent further oxidative modification of unsaturated fatty acids.

Methods

Atherosclerosis Models

Double-knockout gulo−/−/apoE−/− mice that had been backcrossed at least 8 generations onto the C57BL/6 background were obtained from Mutant Mouse Regional Resource Center (MMRRC) (http://www.mmrrc.org). These mice were used in experiment 1, in which vitamins C and E were both modified. In experiment 2, gulo−/−/apoE−/− mice were crossed with SVCT2−/− mice, which had also been separately placed on the C57BL/6 background by 9 backcrosses. Gulo−/−/apoE−/−/SVCT2−/− mice were obtained after backcrossing the resulting SVCT2−/− progeny to gulo−/−/apoE−/− mice. The genotype of each pup was determined by PCR, as previously described, with minor modifications using the following primers: apoE (TGTGACTTGGAGACTCTGCAC, GCGGCCGCCAGTCGCATCC1, and CATTCTCTGCTGCATAGTACG), gulo (CGGCCCTTAATTATAGGATCC, GTCGTGACAGAATGTCTTGC, and CATCTGTGCGTGCATAGTAGC), and SVCT2 (CATTCTGCGCTGTACGACTAAGGAT, and GATGGACGGCATACAAGTGC).

During breeding and pregnancy, all mice received normal rodent chow (Purina Laboratory Diet 5001) that contained about 45 IU/kg vitamin E and negligible amounts of vitamin C. Drinking water for the pregnant dams was changed twice weekly, prepared from deionized water, and contained 0.33 g/L ascorbate and 0.01 mmol/L EDTA. Deionized water was used to prepare the ascorbate-containing drinking water because almost all ascorbate was lost after 48 hours when tap water was used, whereas only about 10% was lost when deionized water was used (data not shown).
Quantification of Arterial Lesions
Mice were killed under general anesthesia, and 30 mL of saline was flushed through the left ventricle. The heart with the proximal aorta intact was removed and embedded in solution (Tissue Tek OCT; Catalog Number 100498-158, VWR Scientific, Suwanee, Ga) and snap frozen in liquid nitrogen. Cryosections (10-μm thickness) of the proximal aorta were prepared as described,22 starting at the aortic sinus and continuing distally according to the method of Paigen et al,23 and adapted for computer analysis.16 Aortic sections were stained with oil red O and counterstained with hematoxylin. Images of 4 sections of each aorta were captured and analyzed with an imaging system (KS 300, release 2.0; Kontron Electronik GmbH, Eching, Germany).

Immunocytochemistry
To detect macrophages, 5-μm serial cryosections of the proximal aorta were fixed in cold acetone and incubated overnight at 4°C in 5-mmol/L phosphate-buffered saline, pH 7.2, with a rat antibody to mouse macrophage–derived foam cells, MOMA-2 (Accurate Chemical and Scientific Corp, Westbury, NY).24 The sections were treated with biotinylated goat antibodies to rat IgG (PharMingen, San Diego, Calif) and incubated with avidin-biotin complex labeled with alkaline phosphatase (Vector Laboratories, Burlingame, Calif). Fast red TR/naphthol AS-MX substrate (Sigma-Aldrich Chemical Co, St Louis, Mo) was used to visualize enzyme activity. Photomicroscopy was performed with a microscope (Zeiss Axiophot) with objectives (Plan-FLUAR). The area of macrophage immunostaining in the atherosclerotic lesions was quantified as previously described for oil red O.

Statistical Analyses
The results were calculated as mean±SD. Statistical significance was determined by ANOVA with post hoc testing (experiment 1) or by nonpaired t testing (experiment 2) using a software program (Sigma Stat 2.0; Jandel Scientific, San Rafael, Calif). Significance was based on P<0.05.

Results

Experiment 1
At the completion of the 8-week experiment, none of the mice showed signs of vitamin E deficiency (ataxia or gait difficulties) or of scurvy (lassitude, changes in fur or grooming, or weight loss). Mouse weights (Table 1) at the end of the diet period were higher in males than females (P=0.002) and higher in females fed high levels of both vitamins. All of the mice had markedly elevated plasma cholesterol levels after 8 weeks of a diet increased in fat and cholesterol (Table 1). Despite the fact that the diets all had the same amount of cholesterol and fat, there were differences among the diets, with varying vitamin contents in both cholesterol and triglycerides. Cholesterol levels in both sexes tended to be higher in males receiving the vitamin E–deficient diets than in mice receiving vitamin E–deficient diets than in mice receiving vitamin E–replete diets (Table 1). In contrast, vitamin E–deficient mice of both sexes had significantly lower triglyceride levels than mice receiving high levels of vitamin E supplements. The presence or absence of vitamin C had no effect on lipid levels, and sex did not have consistent effects across the different treatments.

Ascorbate and α-tocopherol levels in liver and brain cortex were modified as expected, based on the different diets (Table 1). Vitamin levels in brain ascorbate, liver ascorbate, and brain α-tocopherol were modified as expected, based on the different diets (Table 1).
water compared with mice with high vitamin C intakes. The mice with high vitamin C intakes had only approximately normal, not supranormal, plasma and tissue levels of the vitamin. In gulo/H11002/H11002 mice receiving low vitamin C intake for 4 weeks, serum ascorbate levels were 15 ± 4 μmol/L compared with 54 ± 4 μmol/L in littermates fed a high level of vitamin C. This 72% decrease is in line with that seen in the liver. Levels of α-tocopherol in mice receiving low vitamin E diets were decreased by 75% to 90% of the levels in both liver and brain compared with mice receiving high vitamin E diets. In gulo/H11002/H11002 mice receiving a low vitamin E intake for 16 weeks, serum α-tocopherol levels were 3.8 ± 1.4 μmol/L compared with 25 ± 4 μmol/L in littermates fed a high level of vitamin E. This 85% decrease is in line with that seen in the liver. The vitamin E supplement level of 135 IU/kg was somewhat higher than typically supplemented in normal rodent chow (45 to 90 IU/kg). There were no consistent effects of sex or either vitamin E or vitamin C to modify levels of the other vitamin.

To assess oxidative stress in response to vitamin deficiency, F₂-isoprostanes and heart malondialdehyde concentrations were measured in the heart (Figure 1B). There were no sex differences for either measurement; therefore, data from male and female mice were combined. For both measurements, mice with low intakes of both vitamins (E and C) had greater than 50% increases in the levels of both lipid peroxidation products compared with the respective tissues from mice receiving diets supplemented with vitamin E (high E/low C and high E/high C). Even when vitamin C was supplemented to approximately normal levels, a diet low in vitamin E increased both liver F₂-isoprostanes and heart malondialdehyde (low E/high C versus high E/high C) (Figure 1).

The extent of atherosclerosis in the aortic sinus area was determined by measuring the lesion area using oil red O staining for lipids and MOMA immunostaining for macrophages. Lesion size and macrophage content correlated well across the different diets in the individual mice of both sexes (R = 0.55, P < 0.001), supporting the notion that both assays measured the extent of early atherosclerosis, when most of the lesion is composed of viable macrophages. In the male mice, lesion area and macrophage staining were significantly increased when mice received diets low in both vitamins (E and C) (Figure 2A) compared with diets in which vitamin E was supplemented (high E/low C and high E/high C). In the female mice, those fed the diet low in both vitamins (E and C) showed a significantly increased area of macrophage staining with MOMA-2 in the atherosclerotic lesions compared with those fed the diet high in both vitamins (Figure 2B), although the lesion sizes were not significantly different as the result of variability in the high E/high C group. The previously noted tendency for mice with vitamin E deficiency to have higher cholesterol levels did not appear to be the mechanism of the observed effect because lesion area did not show a significant correlation with cholesterol levels in either sex or in the combined sexes (data not shown).

**Experiment 2**

The failure of vitamin C deficiency in affecting atherosclerosis in mice not deficient in vitamin E in the present study and in the study by Maeda et al brings up the question of whether a more severe cellular deficiency of vitamin C might...
have an effect in mice on an adequate intake of vitamin E. To further decrease cellular vitamin C by a nondietary approach, we crossed gulo<sup>−/−</sup>/apoE<sup>−/−</sup> mice with mice hemizygous for deficiency of the ascorbate transporter, SVCT2 (SVCT2<sup>−/−</sup>). These mice were fertile and grew normally when given normal rodent chow and drinking water containing the standard vitamin C supplement of 0.33 g/L. When drinking water containing the low amount of vitamin C used in experiment 1 (0.033 g/L) for 8 weeks, the mice continued to do well and showed no physical signs of scurvy. As shown in Table 2, decreasing the vitamin C intake did not significantly affect the weights of the gulo<sup>−/−</sup>/apoE<sup>−/−</sup> mice. Gulo<sup>−/−</sup>/apoE<sup>−/−</sup>/SVCT2<sup>−/−</sup> mice receiving the same vitamin C and E intakes as the gulo<sup>−/−</sup>/apoE<sup>−/−</sup>/apoE<sup>−/−</sup> mice shown in Table 1 (high E/high C and high E/low C) had decreased heart ascorbate, μmol/g wet weight

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*Data are given as mean±SD (from at least 14 values for each sex and treatment group).
†Values in the same row that do not share superscripts are significantly different (P<0.05).

Discussion

We assessed the impact of combined deficiencies of vitamins C and E on early atherogenesis in apoE-deficient mice unable to synthesize their own vitamin C. Atherosclerosis-prone gulo<sup>−/−</sup>/apoE<sup>−/−</sup> mice showed increased lesion size and macrophage immunostaining in the aortic sinus area after only 8 weeks of a high-fat diet. This regimen resulted in less atherosclerosis (<25×10<sup>3</sup> μm<sup>2</sup>) than that seen by Nakata and Maeda<sup>a</sup> in gulo<sup>−/−</sup>/apoE<sup>−/−</sup> mice receiving normal rodent chow (31×10<sup>3</sup> μm<sup>2</sup> to 35×10<sup>3</sup> μm<sup>2</sup>) for 16 weeks. In male gulo<sup>−/−</sup>/apoE<sup>−/−</sup> mice fed the diet low in vitamins C and E, both the extent of atherosclerosis and the area staining for macrophages within the lesions were increased compared with mice with a high level of supplementation of vitamin E. In female gulo<sup>−/−</sup>/apoE<sup>−/−</sup> mice fed the diet low in vitamins C and E, the area staining for macrophages in the atherosclerotic lesions was significantly increased compared with those fed the diet high in both vitamins, but the trend for an increase in atherosclerosis was not significant. In agreement with Nakata and Maeda<sup>a</sup> there was no effect of moderate vitamin C deficiency in serum and tissues on lesion size when vitamin E levels were not depressed. On the other hand, when a more severe cellular vitamin C deficiency was induced by using gulo<sup>−/−</sup>/apoE<sup>−/−</sup> mice with only half the number of vitamin C transporters (gulo<sup>−/−</sup>/apoE<sup>−/−</sup>/SVCT2<sup>−/−</sup>), the extent of atherosclerosis was increased despite the same relatively high...
level of vitamin E supplementation as in the first experiment. Intracellular ascorbate was decreased by halving the number of ascorbate transporters in the liver and brain and was further decreased when the dietary intake of vitamin C was lowered. The latter was especially evident in the heart, although vitamin C still remained detectable in all 3 tissues. Because these mice did not have signs of scurvy, the results suggest that the atherosclerotic process is differentially sensitive to severe cellular ascorbate depletion. A similar effect of what was likely severe ascorbate deficiency alone was found some years ago in guinea pigs, although vitamin C levels were not measured. The finding that macrophage area was increased in the male gulo−/−/apoE−/−/SVCT2−/− mice on low ascorbate intake suggests that this cell type may play a critical role in the increased susceptibility to atherogenesis with severe deficiency of vitamin C, although further studies at the cellular level are needed to define the mechanism.

In our current studies, combined deficiencies of vitamins C and E were required to increase both atherosclerotic lesion size and macrophage content of the lesions. These results suggest that an adequate intake of vitamin E is more important than that of vitamin C in protecting against atherosclerosis in apoE−/−/gulo−/− mice. Regarding the mechanism of this difference, increased oxidative stress was likely a major contributing factor because lipid peroxidation products were increased in both the heart and liver of the mice receiving vitamin E–deficient diets. Although cholesterol levels also tended to be higher in vitamin E–deficient mice in this study, lesion size did not correlate with cholesterol in either sex, so it seems unlikely that different cholesterol levels contributed to the differences observed.

The role of severe vitamin E deficiency alone was previously evaluated by 2 groups using apoE−/− mice unable to assimilate α-tocopherol as the result of lack of the α-tocopherol transfer protein. After 6 to 7 months of diet treatments, vitamin E deficiency caused a significant 20% to 30% increase in aortic atherosclerosis. In those studies, the vitamin E deficiency required concomitant deficiency of vitamin C. They support the notion that oxidative stress accelerates atherosclerosis and defines the extent to which such vitamins must be depleted to observe an effect on atherosclerosis. Deficiencies to the extent induced in these studies (15% to 28% of normal) are uncommon in humans.

In conclusion, the present results show that both moderate combined vitamin C and E deficiencies and a severe cellular deficiency of vitamin C alone accelerate early atherosclerosis in male apoE−/− mice. They support the notion that oxidative stress accelerates atherosclerosis and defines the extent to which such vitamins must be depleted to observe an effect on atherosclerosis. Deficiencies to the extent induced in these studies (15% to 28% of normal) are uncommon in humans. On the other hand, it is plausible that longer-term but less severe depletion could have similar effects on human atherosclerosis.

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Disclosures
None.

References


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