Long-Term Vitamin C Treatment Increases Vascular Tetrahydrobiopterin Levels and Nitric Oxide Synthase Activity

Livius V. d’Uscio, Sheldon Milstien, Darcy Richardson, Leslie Smith, Zvonimir S. Katusic

Abstract—In cultured endothelial cells, the antioxidant, L-ascorbic acid (vitamin C), increases nitric oxide synthase (NOS) enzyme activity via chemical stabilization of tetrahydrobiopterin. Our objective was to determine the effect of vitamin C on NOS function and tetrahydrobiopterin metabolism in vivo. Twenty-six to twenty-eight weeks of diet supplementation with vitamin C (1%/kg chow) significantly increased circulating levels of vitamin C in wild-type (C57BL/6J) and apolipoprotein E (apoE)–deficient mice. Measurements of NOS enzymatic activity in aortas of apoE-deficient mice indicated a significant increase in total NOS activity. However, this increase was mainly due to high activity of inducible NOS, whereas eNOS activity was reduced. Significantly higher tetrahydrobiopterin levels were detected in aortas of apoE-deficient mice. Long-term treatment with vitamin C restored endothelial NOS activity in aortas of apoE-deficient mice, but did not affect activity of inducible NOS. In addition, 7,8-dihydrobiopterin levels, an oxidized form of tetrahydrobiopterin, were decreased and vascular endothelial function of aortas was significantly improved in apoE-deficient mice. Interestingly, vitamin C also increased tetrahydrobiopterin and NOS activity in aortas of C57BL/6J mice. In contrast, long-term treatment with vitamin E (2000 U/kg chow) did not affect vascular NOS activity or metabolism of tetrahydrobiopterin. In vivo, beneficial effect of vitamin C on vascular endothelial function appears to be mediated in part by protection of tetrahydrobiopterin and restoration of eNOS enzymatic activity. (Circ Res. 2003;92:88-95.)

Key Words: tetrahydrobiopterin ■ nitric oxide synthase ■ nitric oxide ■ antioxidants ■ superoxide anion

Nitric oxide (NO) is a potent vasodilator and plays a key role in control of the cardiovascular system.1 NO is mainly formed in endothelial cells from L-arginine by oxidation of its terminal guanidino-nitrogen,2 requiring the cofactors NADPH, (6R)-5,6,7,8-tetrahydrobiopterin (BH4), FAD, FMN, heme, and Zn2+.4 The formation of NO occurs via endothelial NO-synthase (eNOS) which is expressed constitutively.5,6 Relaxations in response to the abluminal release of endothelial-derived NO are associated with stimulation of soluble guanylyl cyclase (sGC) and in turn formation of cyclic guanosine 3′,5′-monophosphate (cGMP) in vascular smooth muscle cells.7

Inducible NOS (iNOS) enzyme can be expressed in vascular smooth muscle cells, endothelium, and macrophages. This enzyme activity is Ca2+-independent and produces large amounts of NO; it is induced by cytokines such as interleukin 1β and tumor necrosis factor-α and hence is activated in atherosclerosis and inflammatory processes.8–11 BH4 is an essential cofactor required for activity of all NOS isoforms.4,12 During activation of NOS, BH4 is needed for allosteric and redox activation of its enzymatic activity.4,13

Accumulating evidence suggests that alterations in the NO pathway, such as increased NO decomposition by superoxide anion (O2·-) or altered NOS expressions, play a central role in endothelial dysfunction induced by hypercholesterolemia.14 This may be of major importance because NO can substantially inhibit several components of the atherogenic process, such as vascular smooth muscle cells contraction and proliferation, platelet aggregation, and monocyte adhesion.15,16 It has been shown in several studies that antioxidants, vitamin C or vitamin E, reduced vascular oxidative stress17–20 and increased NO-mediated endothelium-dependent relaxations.21,22 In addition, vitamin C increased vasodilation of forearm resistance arteries in humans with hypercholesterolemia,23 long-term smokers,24 essential hypertension,25 and coronary artery disease.26,27 The molecular mechanisms underlying the in vivo antioxidant effects of vitamin C are not fully understood. More recent findings in cultured endothelial cells indicate that vitamin C may increase NOS enzymatic activity by chemical stabilization of BH4.28–30 Therefore, we hypothesized that the in vivo effect of vitamin C is mediated in part by its ability to protect BH4 from oxidation and thereby increase enzymatic activity of eNOS. In this study, we compared the effects of vitamins...
Enzyme Activity

mice.

chemiluminescence as described. 35

of vitamin C and vitamin E were based on previous studies. 18,33

dosages (TD88137, Harlan Teklad)31,32 without or with vitamin C (1%/kg diet) or vitamin E (2000 IU/kg diet) for 26 to 28 weeks. The dosages

L -[ 14 C]-Citrulline formation was measured as described previously. 35

Western Blot Analysis

Mouse monoclonal anti-eNOS (1:500), anti-iNOS (1:100; Transduction Labs), and anti-nitrotyrosine (0.5 μg/mL; Upstate Biotechnol-

ogy) were used. As a loading control, blots were rehybridized with monoclonal anti-actin (Sigma). 33

Measurements of Tissue BH 4 and 7,8-BH 2 /Biopterin

Biopterin levels were determined after differential oxidation in acid and base conditions by reverse-phase HPLC.36–38

Measurements of Intracellular cGMP and cAMP

Radioimmunoassay kits (Amersham) were used to perform the measurements as described elsewhere.37

Calculations and Statistical Analysis

Results are expressed as mean±SEM. Wild-type and apoE-deficient mice groups were compared separately by one-way ANOVA for multiple comparisons. For simple comparisons between two groups, an unpaired Student’s t test was used where appropriate. A value of P<0.05 was considered significant.

An expanded Materials and Methods section can be found in the online data supplement available at http://www.circresaha.org.

Results

Animal Characteristics

Plasma total cholesterol, LDL, and triglyceride concentrations were elevated while HDL levels were reduced in apoE-deficient mice as compared with wild-type mice (P<0.05; Table 1). Concomitant treatment with antioxidant vitamin C or E had no effect on the plasma lipid profile (Table 1). Plasma vitamin C levels were significantly reduced in apoE-deficient mice as compared with wild-type (P<0.05; n=5; Table 1). Conversely, plasma levels of vitamin E were increased in apoE-deficient mice (P<0.05; n=5; Table 1). Supplementation with vitamin C or E increased their concentrations 3- or 2-fold, respectively, in both wild-type and apoE-deficient mice (P<0.05; n=5; Table 1).

Morphology

Aortic lesion areas were significantly reduced by 51% after treatment of apoE-deficient mice with vitamin C (16.7±3.6%; P<0.05 versus apoE group; 34.0±2.7%; n=5). Vitamin E decreased lesion formation by 32% in apoE-deficient mice (data not shown).

Materials and Methods

Experimental Animals

Male C57BL/6J (wild-type) mice and homozygous apoE-deficient mice (4 to 5 weeks old) were obtained from Jackson Laboratory (Bar Harbor, Maine) and were fed a lipid rich Western-type diet (TD88137, Harlan Teklad)31,32 without or with vitamin C (1%/kg diet) or vitamin E (2000 IU/kg diet) for 26 to 28 weeks. The dosages of vitamin C and vitamin E were based on previous studies. 18,33

Housing facilities and all experimental protocols were approved by the Institutional Animal Care and Use Committee of the Mayo Clinic.

Plasma Vitamins C and E

A reverse-phase HPLC was used to determine plasma concentrations of vitamins C and E.

Lesion Assessment

Dissected aortas were opened longitudinally and fixed in 4% buffered paraformaldehyde for 2 hours and were stained in supersaturated Sudan IV solution for an additional 16 hours.34

Vasomotor Reactivity

Isolated aortic rings were connected to a force transducer for recording of isometric force and placed in organ baths filled with 25 mL Krebs solution (37°C; 94% O2/6% CO2; pH 7.4).35 Concentration-dependent response curves to acetylcholine (Ach), and diethylamino (Z)-3-(N,N-diethylnitroso-1-ium-1,2-diolate (DEA-NONOate) were cumulatively obtained during submaximal contractions to phenylephrine.

Quantification of Vascular O2− Production

Vascular O2− production was measured by lucigenin-enhanced chemiluminescence as described.35

Measurement of Ca2+-Dependent NOS

Enzyme Activity

Aortas were homogenized on ice in lysis buffer pH 7.5, and L-[15C]-Citrulline formation was measured as described previously.35

Western Blot Analysis

TABLE 1. Characteristics of ApoE-Deficient and C57BL/6J Mice After 26 to 28 Weeks of Treatment

<table>
<thead>
<tr>
<th>Parameters</th>
<th>C57BL/6J</th>
<th>C57BL/6J+Vit C</th>
<th>C57BL/6J+Vit E</th>
<th>ApoE</th>
<th>ApoE+Vit C</th>
<th>ApoE+Vit E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>6.9±0.6</td>
<td>5.8±0.6</td>
<td>6.9±0.6</td>
<td>22.8±3.7*</td>
<td>22.7±2.9*</td>
<td>22.1±2.8*</td>
</tr>
<tr>
<td>Triglyceride, mmol/L</td>
<td>0.9±0.1</td>
<td>0.6±0.1</td>
<td>0.8±0.1</td>
<td>1.7±0.4*</td>
<td>1.8±0.5*</td>
<td>2.1±0.5*</td>
</tr>
<tr>
<td>LDL, mmol/L</td>
<td>0.9±0.1</td>
<td>0.7±0.1</td>
<td>0.8±0.1</td>
<td>15.8±3.0*</td>
<td>14.3±0.9*</td>
<td>16.2±2.3*</td>
</tr>
<tr>
<td>HDL, mmol/L</td>
<td>5.5±0.6</td>
<td>5.0±0.5</td>
<td>5.9±0.5</td>
<td>2.6±0.3*</td>
<td>2.7±0.1*</td>
<td>3.2±0.3*</td>
</tr>
<tr>
<td>α-tocopherol, μmol/L</td>
<td>114±3</td>
<td>315±42†</td>
<td>ND</td>
<td>88±7†</td>
<td>230±58#</td>
<td>ND</td>
</tr>
<tr>
<td>Aorta</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KCl contraction, g</td>
<td>1.5±0.1</td>
<td>1.5±0.1</td>
<td>1.5±0.1</td>
<td>1.4±0.1</td>
<td>1.3±0.1</td>
<td>1.4±0.1</td>
</tr>
<tr>
<td>Phenylephrine max, %</td>
<td>89±6</td>
<td>86±7</td>
<td>88±5</td>
<td>104±4</td>
<td>105±5</td>
<td>100±4</td>
</tr>
</tbody>
</table>

ApoE indicates apolipoprotein E–deficient mice; C57BL/6J, wild-type mice; ND, not determined; max, maximal response to agonist (percent of 80 mmol/L KCl). Data are mean±SEM of 5 to 12 mice.

*P<0.05 vs C57BL/6J mice (ANOVA + Bonferroni’s); †P<0.05 vs C57BL/6J mice (unpaired t test); and #P<0.05 vs apoE-deficient mice (unpaired t test).

C and E on BH4 and NOS in wild-type and atherosclerotic mice.
Vascular Reactivity

Contractions to 80 mmol/L KCl and concentration-dependent contractions to phenylephrine were not statistically different between apoE-deficient and C57BL/6J mice groups (Table 1).

We have previously shown that in mice aortas, endothelium-dependent relaxation in response to Ach was L-NAME-sensitive. Either vitamin C or E treatment significantly improved NO-mediated endothelium-dependent relaxations to Ach in aortas of apoE-deficient mice (83 ± 2% or 71 ± 3%, respectively; P < 0.05 versus apoE group, maximal relaxation: 59 ± 4%; Figure 1A, right). However, maximal relaxations to Ach were still impaired as compared with C57BL/6J mice (91 ± 1%; P < 0.05). In addition, maximal relaxations to Ach were significantly bigger in vitamin C–treated apoE-deficient mice as compared with mice treated with vitamin E (P < 0.05; Figure 1A, right). In contrast, vitamin C significantly reduced endothelium-dependent relaxations to Ach in wild-type mice (78 ± 3%; P < 0.05), whereas vitamin E did not have any effect (Figure 1A, left).

Endothelium-independent relaxations to the NO donor DEA-NONOate were reduced, and the concentration-response curve was shifted to right in apoE-deficient mice (pD2: 7.4; P < 0.05 versus wild-type mice: 8.5). Vitamin C, but not vitamin E, in part improved the sensitivity to DEA-NONOate in apoE-deficient mice (pD2: 7.7; P < 0.05 versus apoE mice; Figure 1B, right). In contrast, vitamin C reduced relaxations to the NO-donor in wild-type mice (pD2: 8.2; P < 0.05 versus wild-type group: 8.5; Figure 1B, left) without affecting maximal relaxations.

Ca2+-Dependent NOS Activity

In order to evaluate the mechanisms underlying effects of antioxidants on endothelium-dependent relaxations, we measured Ca2+-dependent NOS activity in aortas of apoE-deficient and wild-type mice as determined by conversion of L-[14C]arginine to L-[14C]citrulline in tissue homogenates. Vitamin C selectively increased Ca2+-dependent NOS activity in aortas from both wild-type and apoE-deficient mice (P < 0.05; Figure 2A). Interestingly, vitamin C normalized enzyme activity in apoE-deficient mice to values similar to those found in aortas from wild-type mice. Conversely, vitamin C did not affect eNOS protein expression (Figure 2B; n = 3), whereas vitamin E had no significant effects on eNOS protein expression or NOS activity in either apoE-deficient or wild-type mice (Figure 2).

INOS Enzyme Activity and Protein Expression

In the aortas of wild-type mice, Ca2+-independent NOS activity was very low as compared with Ca2+-dependent NOS activity (P < 0.05; Figures 2A and 3A). iNOS activity was increased in apoE-deficient mice as compared with wild-type (P < 0.05; Figure 3A). In addition, iNOS protein expression was also enhanced in apoE-deficient mice (P < 0.05; Figure 3B). Antioxidant vitamins did not affect iNOS protein expression (Figure 3B). Interestingly, vitamin C selectively increased iNOS enzyme activity in wild-type (P < 0.05; Figure 3A), whereas it had no effect in apoE-deficient mice.

cGMP and cAMP Levels

Basal cGMP levels were reduced in aortas from apoE-deficient mice as compared with wild-type mice (P < 0.05; Figure 4). Vitamin C treatment increased basal cGMP levels only in wild-type (P < 0.05; Figure 4). Basal cAMP levels were not different between wild-type (30 ± 6 pmol/mg) and apoE-deficient mice (25 ± 2 pmol/mg) and after vitamin C treatment (34 ± 4 and 26 ± 3 pmol/mg) or after vitamin E treatment (33 ± 4 and 27 ± 4 pmol/mg), respectively.

Tetrahydrobiopterin Levels

Total aortic biopterin levels were increased in apoE-deficient mice as compared with wild-type mice (P < 0.05). This increase was due to the elevation of BH3 levels (P < 0.05; Figure 5A). 7,8-BH2/biopterin levels were not affected (NS; Figure 5B). The ratios of BH3 to 7,8-BH2/biopterin were not different between two groups of mice (Figure 5C).
Treatment of apoE-deficient mice with vitamin C did not affect aortic BH$_4$ levels. In contrast, vitamin C significantly decreased 7,8-BH$_2$/biopterin levels in apoE-deficient mice (P<0.05; Figure 5B). Conversely, vitamin C significantly increased BH$_4$ levels without affecting on 7,8-BH$_2$/biopterin levels in wild-type mice (P<0.05; Figure 5), whereas vitamin E did not have any effect. Most importantly, vitamin C increased BH$_4$ to 7,8-BH$_2$/biopterin ratio in both apoE-deficient and wild-type mice (P<0.05; Figure 5C).

We also measured BH$_4$ and 7,8-BH$_2$/biopterin levels in the liver in order to determine whether vitamin C may affect BH$_4$ metabolism in tissues other than blood vessel. We found that in wild-type mice, 7,8-BH$_2$/biopterin was very low as compared with BH$_4$ (Table 2). On the other hand, 7,8-BH$_2$/biopterin levels were increased in apoE-deficient mice as compared with wild-type (P<0.05; Table 2). Consequently, BH$_4$ to 7,8-BH$_2$/biopterin ratio decreased in apoE mice (P<0.05). Vitamin C treatment did not have any effect on BH$_4$ and 7,8-BH$_2$/biopterin levels (NS; Table 2), whereas vitamin E slightly decreased BH$_4$ levels in apoE-deficient mice (P<0.05).

![Figure 2](image1.png)

**Figure 2.** A, Bar graphs showing Ca$^{2+}$-dependent eNOS enzyme activity in the aorta of wild-type (C57BL/6J) and apoE-deficient mice after 26 to 28 weeks on a Western-type diet with or without antioxidants. L-[14C]citrulline formation was measured in aortic homogenates as described in Materials and Methods. Results are mean±SEM (n=7). *P<0.05 vs C57BL/6J mice (ANOVA+Bonferroni's); †P<0.05 vs C57BL/6J mice (unpaired t test); #P<0.05 vs apoE-deficient mice (ANOVA+Bonferroni's). B, Representative Western blot analysis of eNOS protein expression in aortas of C57BL/6J and apoE-deficient mice. Bar graph indicates the results of relative densitometric analysis of eNOS expression as OD per mm$^2$ aortic surface (n=3 to 4). Actin blots are shown as loading controls.

![Figure 3](image2.png)

**Figure 3.** A, Bar graphs showing Ca$^{2+}$-independent NOS enzyme activity (iNOS) in the aorta of wild-type (C57BL/6J) and apoE-deficient mice after 26 to 28 weeks on a Western-type diet with or without antioxidants. L-[14C]citrulline formation was measured in aortic homogenates as described in Materials and Methods. Results are mean±SEM (n=7). *P<0.05 vs C57BL/6J mice; †P<0.05 vs C57BL/6J with or without antioxidants (ANOVA+Bonferroni's). B, Representative Western blot analysis of iNOS protein expression in aortas of C57BL/6J and apoE-deficient mice. Bar graphs indicate the results of the relative densitometry as compared with actin (n=3). *P<0.05 vs C57BL/6J with or without antioxidants (ANOVA+Bonferroni's).

![Figure 4](image3.png)

**Figure 4.** Bar graphs showing basal cGMP levels in aortas of wild-type (C57BL/6J) and apoE-deficient mice. Results are mean±SEM (n=5 to 10). *P<0.05 vs C57BL/6J mice; †P<0.05 vs C57BL/6J with or without antioxidants (ANOVA+Bonferroni's).
Vascular $O_2^-$ Production

Formation of $O_2^-$ was increased 3-fold in apoE aortas ($P<0.05$ versus wild-type mice; Figure 6A). Both antioxidant vitamins significantly decreased $O_2^-$ levels in apoE-deficient mice aortas ($P<0.05$ versus apoE group; Figure 6A), whereas they did not affect $O_2^-$ production in wild-type mice.

Detection of Nitrotyrosine

Western blot analysis showed an increased nitrotyrosine abundance in the aorta of apoE-deficient mice (n=4, Figure 6B), whereas in wild-type mice, nitrotyrosine could not be detected (data not shown). Both vitamin C and E reduced tissue nitrotyrosine abundance in apoE-deficient mice (Figure 6B). In order to confirm the specificity of the antibody, sodium dithionite was used to destroy the nitrotyrosine epitope (Figure 6B; lanes 5 to 7).

Discussion

This is the first study to examine in vivo effects of long-term vitamin C treatment on NOS enzymatic activity and BH$_4$ metabolism in aortas of wild-type and apoE-deficient mice.

Table 2. Biopterin Levels in the Liver of ApoE-Deficient and C57BL/6J Mice After 26 to 28 Weeks of Treatment

<table>
<thead>
<tr>
<th>Parameters</th>
<th>C57BL/6J</th>
<th>C57BL/6J + Vit C</th>
<th>C57BL/6J + Vit E</th>
<th>ApoE</th>
<th>ApoE + Vit C</th>
<th>ApoE + Vit E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total liver biopterin, pmol/mg</td>
<td>69.6 ± 9.2</td>
<td>63.5 ± 6.4</td>
<td>66.0 ± 7.7</td>
<td>78.9 ± 5.6</td>
<td>81.8 ± 4.2</td>
<td>64.2 ± 5.4</td>
</tr>
<tr>
<td>BH$_4$, pmol/mg</td>
<td>64.1 ± 6.3</td>
<td>58.1 ± 6.0</td>
<td>58.3 ± 6.1</td>
<td>65.5 ± 5.5</td>
<td>71.4 ± 3.9</td>
<td>50.1 ± 4.0†</td>
</tr>
<tr>
<td>7,8-BH$_2$/biopterin, pmol/mg</td>
<td>5.5 ± 0.9</td>
<td>5.3 ± 1.1</td>
<td>7.7 ± 2.0</td>
<td>13.4 ± 1.6*</td>
<td>10.2 ± 1.3*</td>
<td>14.1 ± 2.6*</td>
</tr>
<tr>
<td>BH$_4$/7,8-BH$_2$/biopterin ratio</td>
<td>11.8 ± 1.8</td>
<td>10.9 ± 2.5</td>
<td>7.6 ± 1.4</td>
<td>4.9 ± 0.8*</td>
<td>7.0 ± 0.9</td>
<td>3.5 ± 0.6</td>
</tr>
</tbody>
</table>

ApoE indicates apolipoprotein E–deficient mice; C57BL/6J, wild-type mice; BH$_4$, tetrahydrobiopterin; 7,8-BH$_2$, 7,8-dihydrobiopterin.

Data are mean ± SEM of 8 to 9 mice.

*P<0.05 vs C57BL/6J mice (unpaired t test); †P<0.05 vs apoE group (ANOVA + Bonferroni’s).

Figure 6. A, Detection of superoxide anion in mouse aortas by lucigenin-enhanced chemiluminescence. Photon counts were averaged over 8 minutes and were expressed as counts per min per μg dry weight. Results are mean ± SEM (n = 7 to 9). *P<0.05 vs C57BL/6J mice; †P<0.05 vs apoE-deficient mice (ANOVA + Bonferroni’s). B, Representative Western blot analysis of nitrotyrosine abundance in aortas of apoE-deficient mice. Lane 1 corresponds to 3 μmol/L nitrated bovine albumin as a positive control. Lanes 2 and 5 are apoE; lanes 3 and 6, apoE + vitamin C; and lanes 4 and 7, apoE + vitamin E groups. Lanes 5 to 7, Sodium dithionite (20 mmol/L) treatment (n=3 experiments). Actin blot is shown as loading control.
We report a number of novel findings. First, vitamin C treatment increased total biopterin and BH₄ levels in aorta of wild-type mice. This increase was associated with increased enzymatic activity of eNOS, iNOS, and higher basal levels of cGMP, suggesting that vitamin C has a BH₄-dependent stimulatory effect on NO formation in normal arterial wall. Second, total biopterin, BH₄, and iNOS enzymatic activity were significantly higher in apoE-deficient mice as compared with wild-type mice. Third, supplementation with vitamin C improved endothelial dysfunction in apoE-deficient mice, reduced atherosclerotic lesions, and restored eNOS enzymatic activity. This is most likely due, in part, to the ability of vitamin C to protect BH₄ and to preserve biosynthesis of NO. Fourth, in contrast to vitamin C, vitamin E did not affect vascular NOS enzymatic activity or BH₄ metabolism. Thus, our results demonstrate that vitamin C (but not vitamin E) is an important regulator of BH₄ metabolism and NOS function in vivo.

BH₄ is an essential cofactor required for activity of NOS. Previous studies in cultured vascular endothelial cells demonstrated that vitamin C increases eNOS activity by increasing availability of BH₄. Increased availability of BH₄ was not due to higher activity of GTP cyclohydrolase I, the rate-limiting enzyme in biosynthesis of BH₄. Rather, chemical stabilization of BH₄ by vitamin C may be the most likely explanation for previously reported observations. In the present study, we tested this concept in vivo by long-term dietary supplementation of vitamin C. Our findings support the idea that vitamin C may increase intracellular concentrations of BH₄ in the normal arterial wall. This, in turn, may activate NOS and increase formation of NO. Increased enzymatic activity of NOS and higher cGMP (but not cAMP) levels in arteries obtained from vitamin C–treated wild-type mice strongly suggest that formation of NO is selectively augmented by vitamin C treatment. It is interesting that iNOS is expressed in wild-type mouse arteries and its activity is very low as compared with Ca²⁺–dependent NOS activity. The fact that vitamin C did not affect expression of eNOS or iNOS protein, together with a significant increase in eNOS and iNOS enzymatic activity, suggest that availability of BH₄ may be a regulatory mechanism designed to control levels of NO production. It appears that in vivo intracellular concentration of BH₄ is subsaturating for vascular NOS isoforms.

Endothelium-dependent relaxations to Ach and endothelium-independent relaxations to DEA-NONOate were impaired in the aortas of vitamin C–treated wild-type mice. This finding is consistent with reported impairment of NO-induced relaxation in eNOS transgenic mice and arteries transduced with recombinant iNOS. Vitamin C did not increase formation of O₂⁻ in normal arteries, ruling out chemical antagonism between O₂⁻ and NO as an explanation for impairment of relaxations mediated by NO. Downregulation of expression and function of soluble guanylate cyclase in vitamin C–treated aortas is the most likely reason behind reduced reactivity of vascular smooth muscle to NO. Further studies are needed to determine the exact mechanism responsible for reduction of relaxations induced by endogenous or exogenous NO. Our results also call for further studies of BH₄ catabolism in normal arteries. Turnover of BH₄ in blood vessels appears to be very rapid. In isolated canine basilar arteries, incubation with a GTP cyclohydrolase I inhibitor for 6 hours resulted in 95% depletion of intracellular BH₄. The exact molecular mechanisms responsible for degradation of BH₄ that can be inhibited by vitamin C remain to be determined.

Proinflammatory cytokines, including tumor necrosis factor-α, interferon-γ, and interleukin-1β, stimulate BH₄ biosynthesis in cultured vascular endothelial cells. This effect is due to upregulation of GTP cyclohydrolase I transcription, expression, and function. Simmons and colleagues demonstrated that in cardiac microvascular endothelial cells cytokines cause coordinate induction of GTP cyclohydrolase I and iNOS. Cytokines play a key role in pathogenesis of atherosclerosis, and therefore, it is not surprising that in the present study we detected 2-fold increases of BH₄ in aortas of apoE-deficient mice. This increase in BH₄ was associated with about 7-fold increase in iNOS enzymatic activity. Thus, the present in vivo findings are consistent with previously obtained results in cultured endothelial cells and support the hypothesis that biosynthesis of BH₄ is coordinated with induction and increased activity of iNOS. They are also consistent with reported increased plasma levels of neopterin, a by-product of BH₄ biosynthesis, in patients with atherosclerosis and coronary syndromes.

In apoE-deficient mice, vitamin C treatment did not affect aortic BH₄ levels, but did significantly reduce the BH₄ fraction, suggesting that vitamin C may protect BH₄ from oxidation. Catabolism of BH₄ has not been studied in apoE-deficient mice, and we can only speculate about molecular mechanisms underlying protection of BH₄. In a previous study, we demonstrated that peroxynitrite causes oxidation of BH₄. This has been confirmed in two subsequent reports. Whether endogenous peroxynitrite contributes to oxidation of BH₄ in vivo is unknown. Vitamin C could lessen redox cycling of BH₄ by decreasing intracellular O₂⁻ and peroxynitrite accumulation because BH₄ has been shown to undergo redox cycling with molecular oxygen, which results in the generation of O₂⁻. However, because both vitamins C and E reduced production of O₂⁻ and nitrotyrosine, but only vitamin C had effects on BH₄ and NOS activity, it appears unlikely that O₂⁻/peroxynitrite-mediated oxidation is responsible for oxidation of BH₄. Furthermore, vitamin C was very effective in increasing BH₄ levels in wild-type animals despite the absence of nitrotyrosine and very low O₂⁻ formation in their aortas. Studies in cultured vascular endothelial cells demonstrated that oxidation of BH₄ to quinonoid 6,7-[8H]-BH₂ or BH₃ radical to BH₄ radical is responsible for oxidation of BH₄. In addition, vitamin C could also increase the affinity of BH₄ for NOS enzyme by preserving thiols on NOS that are required for binding of the cofactor and, in turn, may stimulate NO-production.

Despite the fact that mice have ability to synthesize vitamin C (unlike humans), increased dietary intake of vitamin C stimulated NOS enzymatic activity in wild-type...
and apoE-deficient mice. It is possible that the high level of oxidative stress that was found in atherosclerotic apoE-deficient mice may consume vitamin C. Indeed, plasma concentrations of vitamin C were significantly lower in apoE-deficient mice. This is consistent with results of epidemiological studies in humans demonstrating that plasma vitamin C concentrations are inversely related to increased risk for atherosclerosis. Thus, supplementation of vitamin C may help to replace oxidized vitamin C in apoE-deficient mice. Why long-term treatment with vitamin C increases NOS activity in wild-type animals is unclear and remains to be determined.

Our study is the first to examine the effect of vitamin C on endothelial dysfunction and progression of atherosclerosis in apoE-deficient mice. As expected, vitamin C improved endothelial function, and reduced O2- and peroxynitrite formation. These effects could be independent of the effect of vitamin C on BH4 metabolism. Endothelial cells can take up reduced or oxidized forms of ascorbic acid and accumulate concentrations up to 3 to 8 mmol/L. This concentration of vitamin C can effectively scavenge O2- and protect NO from chemical inactivation. With regard to the antiatherogenic effect of vitamin E, our results are in agreement with the previously reported ability of vitamin E to prevent development of atherosclerosis in apoE-deficient mice. The present study demonstrates that long-term treatment of C57BL/6J mice with vitamin C increases BH4 levels in the vascular wall. This increase is coupled with increased eNOS enzymatic activity and high basal levels of cGMP. We also provide evidence that BH4 metabolism may be an important component in pathogenesis of atherosclerosis. Coordinated upregulation of BH4 availability and iNOS expression is probably designed to increase biosynthesis of NO in vascular wall exposed to proinflammatory cytokines. However, prolonged high activity of iNOS may be detrimental to vascular function due to “uncoupling” of the enzyme and subsequent increased formation of O2-.

Protection of BH4 appears to be an important mechanism that may contribute to antiatherogenic effect of vitamin C.

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Long-Term Vitamin C Treatment Increases Vascular Tetrahydrobiopterin Levels and Nitric Oxide Synthase Activity
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Expanded Materials and Methods for On-line Publication

*Experimental Animals:* Male C57BL/6J (control) mice and homozygous apoE-deficient mice (4-5 weeks) were obtained from Jackson Laboratory. Housing facilities and all experimental protocols were approved by the Institutional Animal Care and Use Committee of the Mayo Clinic. Control and apoE-deficient mice were fed a lipid rich Western-type diet (0.15% cholesterol and 42% of milk fat by weight, TD88137, Harlan Teklad)\(^1,2\) without or with vitamin C (1%/kg diet) or vitamin E (2000 I.U./kg diet) for 26-28 weeks. Special diets were prepared by Harlan Teklad and were stored under exclusion of O\(_2\) in a 4\(^\circ\)C cold room. The dosages of vitamin C and vitamin E were based on previous studies.\(^3,4\)

The mice were anesthetized (pentobarbital, 60 mg/kg body weight, i.p.) and sacrificed. Whole aortas were carefully removed and dissected free from connective tissue in cold (4\(^\circ\)C) modified Krebs-Ringer bicarbonate solution (in mmol/L: NaCl 118.6; KCl 4.7; CaCl\(_2\) 2.5; MgSO\(_4\) 1.2; KH\(_2\)PO\(_4\) 1.2; NaHCO\(_3\) 25.1; glucose 10.1; EDTA 0.026).

*Plasma Lipid Profile:* Blood samples were obtained through puncture of the right ventricle. Blood was immediately transferred to a tube containing heparin and centrifuged at 4\(^\circ\)C for 10 minutes. Plasma was stored at -80\(^\circ\)C until assayed. Cholesterol, HDL and triglycerides were determined using a colorimetric-based assay on a Cobas Mira\(^8\) Systems.\(^5\)

*Plasma Vitamins C and E:* For vitamin C, 10% meta-phosphoric acid was used to remove proteins and stabilize the L-ascorbic acid which was then analyzed by UV absorbance after elution from a reverse-phase HPLC column with phosphate buffer (pH 2).\(^6\) For vitamin E, the proteins were precipitated with ethanol and vitamin E extracted with heptane. An aliquot was analyzed by reverse-phase HPLC eluted with 75:25 acetonitrile:MeOH and quantitated with a photodiode array detector.\(^7\)

*Lesion Assessment:* Dissected aortas were opened longitudinally and fixed in 4% buffered paraformaldehyde for 2 hrs. Lipid-rich intraluminal lesions were stained in
supersaturated Sudan IV solution in 38% isopropanol for an additional 16 hrs. After staining, aortas were washed in distilled water and kept in 4% formalin. Serial images of the submerged vessels were captured with a digital camera. Lesion analysis was performed with Image Pro Plus® 3.0 software. The amount of aortic lesion formation in each animal was measured as % lesion area per total area of the aorta.

**Vasomotor Reactivity:** Aortas were cut into rings (4 mm) from the distal thoracic and/or first part of the abdominal aorta as described. Isolated aorta rings from C57BL/6J, C57BL/6J + vitamin C, and C57BL/6J + vitamin E groups or apoE, apoE + vitamin C, and apoE + vitamin E groups were studied in parallel. Rings were connected to a force transducer (Gould Instrument Systems, Valley View, OH) for recording of isometric force and placed in organ baths filled with 25 mL Krebs solution (37°C; 94% O₂/6% CO₂; pH 7.4). After an equilibration period of 30 min, rings were progressively stretched to their optimal passive force of 1.6 ± 0.1 g as assessed by the response to 80 mmol/L KCl.

Concentration-dependent response curves to acetylcholine (Ach; 10⁻⁹-10⁻⁵ mol/L), and diethylammonium (Z)-1-(N,N-diethylamino)diazene-1,2-diolate (DEA-NONOate; 10⁻¹⁰-10⁻⁵ mol/L) were cumulatively obtained during submaximal contractions to phenylephrine. Concentrations of phenylephrine (1-5 x10⁻⁷ mol/L) were selected in order to obtain the same submaximal contraction in aortic rings from both apoE-deficient and C57BL/6J mice. In addition, concentration-dependent response curves to phenylephrine (10⁻⁹-10⁻⁵ mol/L) were constructed.

**Drugs:** Ach hydrochloride, and phenylephrine were from Sigma Chemical Co. DEA-NONOate was from Cayman Chemical. All drugs were dissolved in distilled water, except DEA-NONOate, which was prepared as stock solutions in 1.5 mol/L Tris, pH 8.8. All drugs were then diluted in Krebs solution and concentrations are expressed as final molar concentration (mol/L) in the organ bath.
**Quantification of Vascular O$_2^-$ Production:** Vascular O$_2^-$ production was measured by lucigenin-enhanced chemiluminescence. A low concentration of lucigenin (5 µmol/L) was used to avoid autoxidation of lucigenin. Briefly, aortas were cut in 3 mm segments and equilibrated in a modified Krebs-Hepes buffer (37°C; pH 7.4; 30 minutes). Scintillation vials containing 2 mL Krebs-Hepes buffer with lucigenin were placed into a liquid scintillation spectrometer (LS 5000, Beckman Instruments Inc., Fullerton, CA) switched to the out-of-coincidence mode. After dark adaptation, background signals were recorded and vascular rings were then added to the vials. Photon counts were recorded every 2 minutes for 8 minutes, and the background was subtracted. The vessels were then dried for 24 hrs at 90°C and weighed. The results were expressed as counts per min per µg dry weight.

**Measurement of Ca$^{2+}$-Dependent NOS Enzyme Activity:** Aortas from 3 different control or 3 different apoE-deficient mice groups were analyzed in parallel. Briefly, fresh aortas were homogenized on ice in lysis buffer (pH 7.5) containing 50 mmol/L Tris-HCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 0.1% SDS, 0.1% deoxycholate, 1% IGEPA L, and mammalian protease inhibitor cocktail (all from Sigma). The homogenates were then sonicated 30 s, rotated 1 hr at 4°C, and centrifuged to remove insoluble matter. Total protein was determined using the BioRad DC Protein assay kit (Hercules, CA). L-[14C]-Citrulline formation was measured as described. For total NOS activity, 100 µg protein was added to reaction cocktails containing 1 mmol/L NADPH, 100 nmol/L calmodulin, 30 µM BH$_4$, 2.5 mmol/L CaCl$_2$ and 40 µM L-[U-14C]arginine (348 mCi/mmol, Amersham Life Science). Ca$^{2+}$-independent NOS activity was measured under identical conditions omitting the CaCl$_2$ and adding 2 mmol/L EDTA. Ca$^{2+}$-dependent NOS enzyme activity was calculated by subtracting Ca$^{2+}$-independent NOS activity from total NOS activity. Backgrounds were incubated under same conditions but in the absence of protein.

**Western Blot Analysis:** Equal amounts of protein (100 µg/lane for iNOS and nitrotyrosine) or equal surface areas of aortas (≈50 µg for eNOS) from all groups were
separated by SDS-PAGE and transferred to nitrocellulose membrane (Amersham) using a semi-dry electrophoretic transfer cell for Western analysis. Mouse monoclonal anti-eNOS (1:500), anti-iNOS (1:100; Transduction Labs), and anti-nitrotyrosine (0.5 µg/mL; Upstate Biotechnology Inc.) were used. As a loading control, blots were rehybridized with monoclonal anti-actin (1:25,000; Sigma). Bands were visualized by ECL using a commercially available kit (Amersham Life Science). Densitometry was carried out using NIH Image® (Version 1.6.1.) and the results were expressed as O.D. per mm² aortic surface (eNOS) or relative to the respective intensity of the actin blot (iNOS).

For anti-nitrotyrosine studies, 0.2% bovine albumin in PBS (Gibco) was treated with 2 mM peroxynitrite (Upstate) for 30 minutes at 37°C and this was used as a positive control. To control for the specificity of the antibody, aorta homogenates were incubated with fresh sodium dithionite solution (20 mM final concentration) for 30 minutes.12

**Measurements of Tissue BH₄ and 7,8-BH₂/Biopterin:** Fresh Aortas and livers were homogenized in buffer containing 50 mmol/L Tris (pH 7.4), 1 mmol/L dithiothreitol, and 1 mmol/L EDTA at 4°C and were centrifuged at 12,000 g (10 minutes at 4°C). Biopterin levels were determined after differential oxidation in acid (which converts both BH₄ and 7,8-BH₂ to biopterin) and base (which converts only 7,8-BH₂ to biopterin) conditions by reverse-phase HPLC as described previously.13-15 BH₄ content was calculated from the difference in biopterin levels after acid and base oxidations.

**Measurements of Intracellular cGMP and cAMP:** 10-15 mm of aorta was incubated for 30 min in MEM (with Earle's salts containing 0.1% BSA, 100 U/mL penicillin and 100 µg/mL streptomycin) with 3-isobutyl-1-methylxanthine (10⁻⁴ mol/L) to inhibit the degradation of cyclic nucleotides by phosphodiesterases. Aortas were then removed and frozen in liquid N₂ until assayed. cGMP and cAMP radioimmunoassay kits (Amersham Life Science) were used to perform the measurements as described elsewhere.15 The results were expressed as pmol/mg protein.
**Calculations and Statistical Analysis:** Results are expressed as means ± SEM and n indicates the number of animals from which tissues were harvested. Contractions were expressed as percent of the response to a second KCl (80 mmol/L) induced contraction, which was obtained at the beginning of each experiment. Maximal relaxation or contraction and was determined for each individual concentration-response curve by non-linear regression analysis. Median effective concentration (EC$_{50}$) was calculated and expressed as its negative logarithm (pD$_2$). Statistical analysis of concentration-response curves of the different groups was compared by ANOVA for repeated measurements followed by Bonferroni's correction. Control and apoE-deficient mice groups were compared separately by one-way ANOVA for multiple comparisons. For simple comparisons between two groups, an unpaired Student's t-test was used where appropriate. A value of P<0.05 was considered significant.

**References**


