

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

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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⁺.^{3,4} The formation of NO occurs via endothelial NO-synthase (eNOS) which is expressed constitutively.^{5,6} Relaxations in response to the abluminal release of endothelium-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 1 β 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

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

Parameters	C57BL/6J	C57BL/6J+Vit C	C57BL/6J+Vit E	ApoE	ApoE+Vit C	ApoE+Vit E
Plasma						
Total cholesterol, mmol/L	6.9±0.6	5.8±0.6	6.9±0.6	22.8±3.7*	22.7±2.9*	22.1±2.8*
Triglyceride, mmol/L	0.9±0.1	0.6±0.1	0.8±0.1	1.7±0.4*	1.8±0.5*	2.1±0.5*
LDL, mmol/L	0.9±0.1	0.7±0.1	0.8±0.1	15.8±3.0*	14.3±0.9*	16.2±2.3*
HDL, mmol/L	5.5±0.6	5.0±0.5	5.9±0.5	2.6±0.3*	2.7±0.1*	3.2±0.3*
L-ascorbic acid, μmol/L	114±3	315±42†	ND	88±7†	230±58#	ND
α-tocopherol, μmol/L	35±2	ND	47±3†	49±4†	ND	98±8#
Aorta						
KCl contraction, g	1.5±0.1	1.5±0.1	1.5±0.1	1.4±0.1	1.3±0.1	1.4±0.1
Phenylephrine max, %	89±6	86±7	88±5	104±4	105±5	100±4

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.

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 super-saturated 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 diethylammonium (Z)-1-(N,N-diethylamino)diazen-1-ium-1,2-diolate (DEA-NONOate) were cumulatively obtained during sub-maximal 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-[¹⁴C]-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).³⁵

Measurements of Tissue BH₄ and 7,8-BH₂/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).

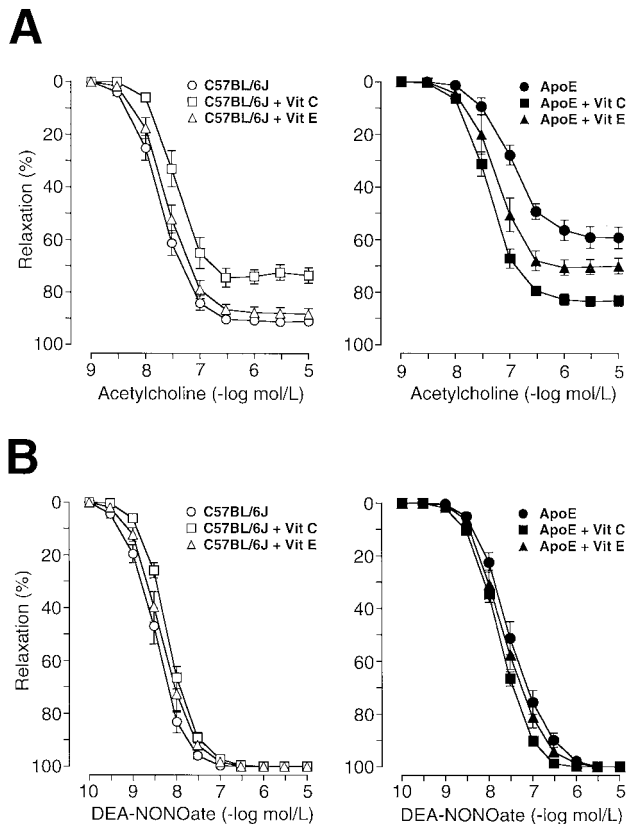


Figure 1. Effects of antioxidants on endothelium-dependent relaxations to Ach and endothelium-independent relaxations to DEA-NONOate in the aorta of wild-type (C57BL/6J) and apoE-deficient mice after 26 to 28 weeks on a Western-type diet. A, Both vitamin C and E improved relaxations to Ach in apoE-deficient mice while, conversely, vitamin C impaired these relaxations ($P < 0.05$; $n = 9$ to 12; ANOVA+Bonferroni's). Note that relaxations to Ach were still impaired in vitamin C-treated apoE-deficient mice as compared with C57BL/6J mice ($P < 0.05$). B, Only vitamin C significantly improved relaxations to DEA-NONOate in apoE-deficient mice while, conversely, vitamin C impaired these relaxations in C57BL/6J mice ($P < 0.05$; $n = 8$ to 12; ANOVA+Bonferroni's). Note that relaxations were still impaired in vitamin C-treated apoE-deficient mice as compared with wild-type mice ($P < 0.05$; ANOVA+Bonferroni's). Results are mean \pm SEM and expressed as percent relaxation of the sub-maximal contraction to phenylephrine (1 to 6×10^{-7} mol/L).

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.³⁵ Either vitamin C or E treatment significantly improved NO-mediated endothelium-dependent relaxations to Ach in aortas of apoE-deficient mice ($83 \pm 2\%$ or $71 \pm 3\%$, respectively; $P < 0.05$ versus apoE group, maximal relaxation: $59 \pm 4\%$; Figure 1A, right). However, maximal relaxations to Ach were still impaired as compared with C57BL/6J mice ($91 \pm 1\%$; $P < 0.05$). 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 ($78 \pm 3\%$; $P < 0.05$), 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 BH₄ levels ($P < 0.05$; Figure 5A). 7,8-BH₂/biopterin levels were not affected (NS; Figure 5B). The ratios of BH₄ to 7,8-BH₂/biopterin were not different between two groups of mice (Figure 5C).

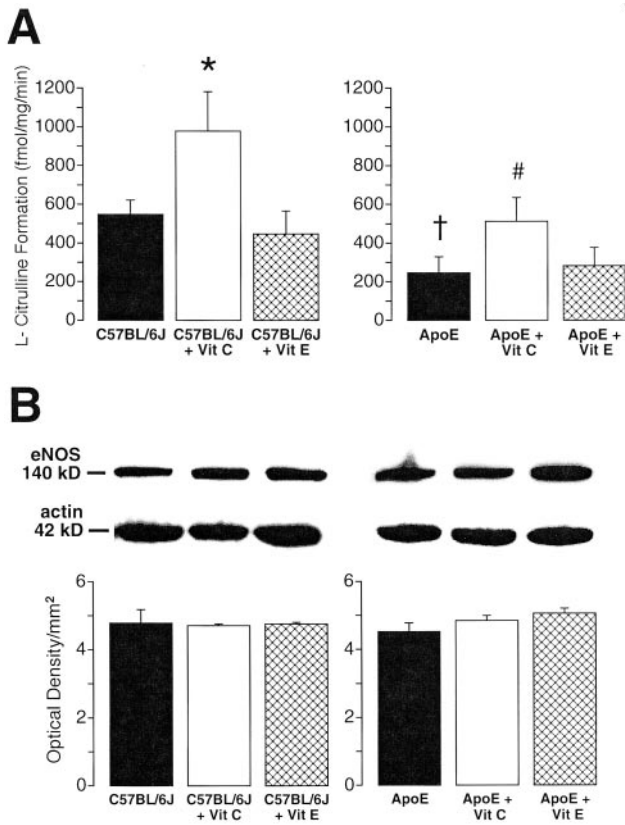


Figure 2. A, Bar graphs showing Ca²⁺-dependent eNOS enzyme activity in the aorta of wild-type (C57BL/6J) and apoE-deficient mice after 26 to 28 weeks on a Western-type diet with or without antioxidants. L-[¹⁴C]citruilline formation was measured in aortic homogenates as described in Materials and Methods. 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 mm² aortic surface (n=3 to 4). Actin blots are shown as loading controls.

Treatment of apoE-deficient mice with vitamin C did not affect aortic BH₄ levels. In contrast, vitamin C significantly decreased 7,8-BH₂/biopterin levels in apoE-deficient mice (P<0.05; Figure 5B). Conversely, vitamin C significantly increased BH₄ levels without affecting on 7,8-BH₂/biopterin levels in wild-type mice (P<0.05; Figure 5), whereas vitamin E did not have any effect. Most importantly, vitamin C increased BH₄ to 7,8-BH₂/biopterin ratio in both apoE-deficient and wild-type mice (P<0.05; Figure 5C).

We also measured BH₄ and 7,8-BH₂/biopterin levels in the liver in order to determine whether vitamin C may affect BH₄ metabolism in tissues other than blood vessel. We found that in wild-type mice, 7,8-BH₂/biopterin was very low as compared with BH₄ (Table 2). On the other hand, 7,8-BH₂/biopterin levels were increased in apoE-deficient mice as compared with wild-type (P<0.05; Table 2). Consequently, BH₄ to 7,8-BH₂/biopterin ratio decreased in apoE mice

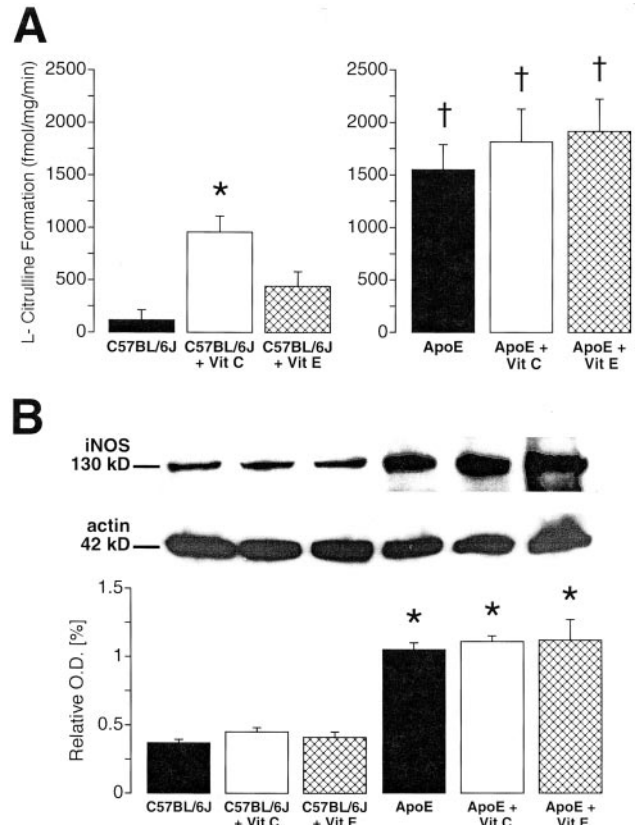


Figure 3. A, Bar graphs showing Ca²⁺-independent NOS enzyme activity (iNOS) in the aorta of wild-type (C57BL/6J) and apoE-deficient mice after 26 to 28 weeks on a Western-type diet with or without antioxidants. L-[¹⁴C]citruilline formation was measured in aortic homogenates as described in Materials 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).

(P<0.05). Vitamin C treatment did not have any effect on BH₄ and 7,8-BH₂/biopterin levels (NS; Table 2), whereas vitamin E slightly decreased BH₄ levels in apoE-deficient mice (P<0.05).

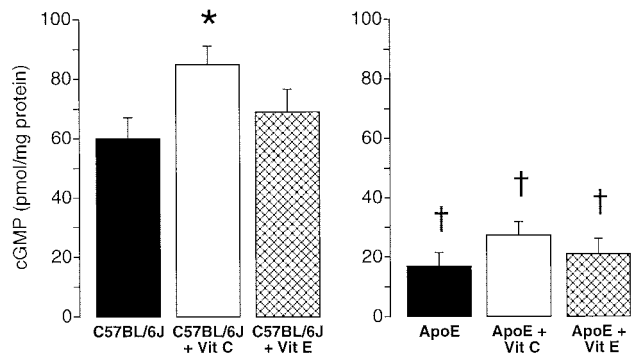


Figure 4. Bar graphs showing basal cGMP levels in aortas of wild-type (C57BL/6J) and apoE-deficient mice. Results are mean ± SEM (n=5 to 10). *P<0.05 vs C57BL/6J mice; †P<0.05 vs C57BL/6J with or without antioxidants (ANOVA+Bonferroni's).

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

Parameters	C57BL/6J	C57BL/6J+Vit C	C57BL/6J+Vit E	ApoE	ApoE+Vit C	ApoE+Vit E
Total liver biopterin, pmol/mg	69.6±9.2	63.5±6.4	66.0±7.7	78.9±5.6	81.6±4.2	64.2±5.4
BH ₄ , pmol/mg	64.1±8.8	58.1±6.0	58.3±6.1	65.5±5.5	71.4±3.9	50.1±4.0†
7,8-BH ₂ +biopterin, pmol/mg	5.5±0.9	5.3±1.1	7.7±2.0	13.4±1.6*	10.2±1.3*	14.1±2.6*
BH ₄ /(7,8-BH ₂ +biopterin) ratio	11.8±1.8	10.9±2.5	7.6±1.4	4.9±0.8*	7.0±0.9	3.5±0.6

ApoE indicates apolipoprotein E-deficient mice; C57BL/6J, wild-type mice; BH₄, tetrahydrobiopterin; 7,8-BH₂, 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).

Vascular O₂⁻ Production

Formation of O₂⁻ was increased 3-fold in apoE aortas (*P*<0.05 versus wild-type mice; Figure 6A). Both antioxidant vitamins significantly decreased O₂⁻ levels in apoE-deficient mice aortas (*P*<0.05 versus apoE group; Figure 6A), whereas they did not affect O₂⁻ 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 BH₄ metabolism in aortas of wild-type and apoE-deficient mice.

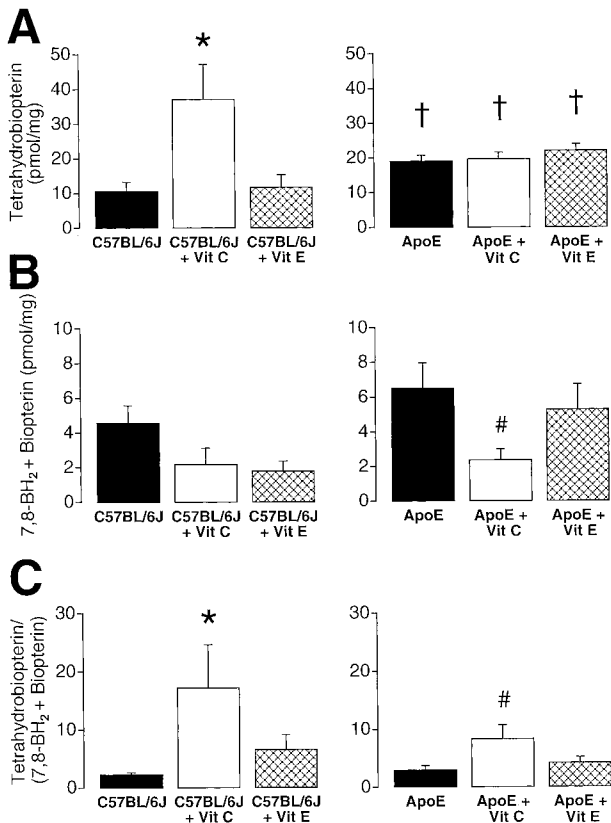


Figure 5. Bar graphs showing BH₄ levels (A), 7,8-BH₂/biopterin levels (B), and BH₄ to 7,8-BH₂/biopterin ratio (C) in aortas of wild-type (C57BL/6J) and apoE-deficient mice after 26 to 28 weeks on a Western-type diet with or without antioxidants. Results are mean±SEM (n=4 to 7). **P*<0.05 vs wild-type mice (ANOVA+Bonferroni's); †*P*<0.05 vs C57BL/6J mice (unpaired *t* test); #*P*<0.05 vs apoE-deficient mice (ANOVA+Bonferroni's).

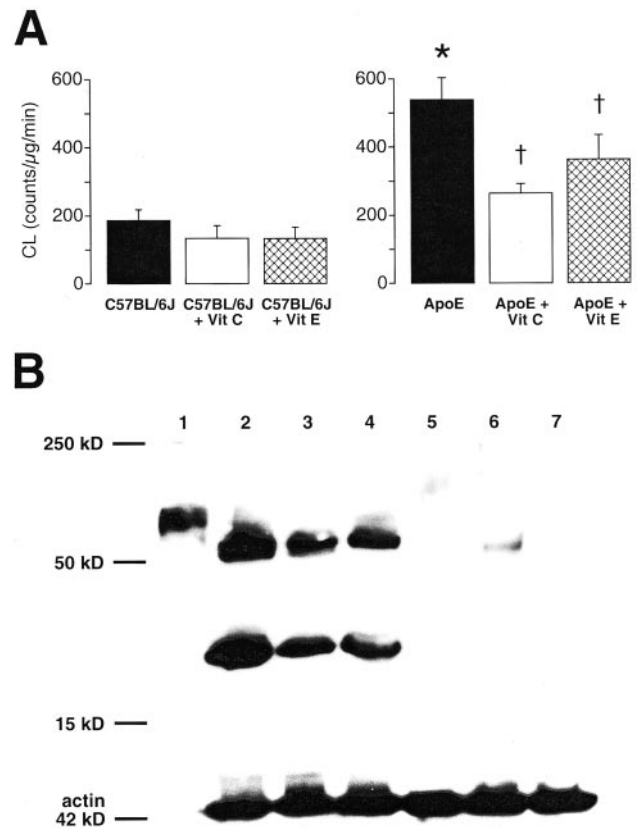


Figure 6. A, Detection of superoxide anion in mouse aortas by lucigenin-enhanced chemiluminescence. Photon counts were averaged over 8 minutes and were expressed as counts per min per μg dry weight. Results are mean±SEM (n=7 to 9). **P*<0.05 vs C57BL/6J mice; †*P*<0.05 vs apoE-deficient mice (ANOVA+Bonferroni's). 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.⁶ Previous studies in cultured vascular endothelial cells demonstrated that vitamin C increases eNOS activity by increasing availability of BH₄.^{28–30} 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.^{39,40} 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.^{39,41} 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 BH₄.⁴² 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-1 β , stimulate BH₄ biosynthesis in cultured vascular endothelial cells.^{43–46} This effect is due to upregulation of GTP cyclohydrolase I transcription, expression, and function.⁴⁶ Simmons and colleagues demonstrated that in cardiac microvascular endothelial cells cytokines cause coordinate induction of GTP cyclohydrolase I and iNOS.⁴⁵ 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.^{47,48}

In apoE-deficient mice, vitamin C treatment did not affect aortic BH₄ levels, but did significantly reduce the BH₂ 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 BH₄.³⁸ This has been confirmed in two subsequent reports.^{49,50} 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⁻.⁵¹ 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, rearrangement to 7,8-BH₂ and further oxidation to biopterin is most likely the main pathway of BH₄ degradation.³⁰ Based on the results obtained in cultured endothelium, the stabilizing effect of vitamin C may be due to a chemical reduction of quinonoid 6,7-(8)-BH₂ or BH₃ radical to BH₄.⁵² 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.^{28,30,53}

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^{35,49} 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.^{54–57} 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.⁵⁸ This concentration of vitamin C can effectively scavenge O₂⁻ and protect NO from chemical inactivation.⁵⁹ 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.¹⁸

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⁻.⁶⁰ Protection of BH₄ appears to be an important mechanism that may contribute to antiatherogenic effect of vitamin C.

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References

- Lüscher TF, Vanhoutte PM. *The Endothelium: Modulator of Cardiovascular Function*. Boca Raton, Fla: CRC Press; 1990.
- Palmer RMJ, Ashton DS, Moncada S. Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature*. 1988;333:664–666.
- Ignarro LJ. Biosynthesis and metabolism of endothelium-derived nitric oxide. *Ann Rev Pharmacol Toxicol*. 1990;30:535–560.
- Raman CS, Li H, Martasek P, Kral V, Masters BS, Poulos TL. Crystal structure of constitutive endothelial nitric oxide synthase: a paradigm for pterin function involving a novel metal center. *Cell*. 1998;95:939–950.
- Förstermann U, Schmitt HHHW, Pollock JS, Sheng H, Mitchell JA, Warner TD, Nakane M, Murad F. Isoforms of nitric oxide synthase: characterization and purification from different cell types. *Biochem Pharmacol*. 1991;42:1849–1857.
- Pollock JS, Förstermann U, Mitchell JA, Warner TD, Schmidt HH, Nakane M, Murad F. Purification and characterization of particulate endothelium-derived relaxing factor synthase from cultured and native bovine aortic endothelial cells. *Proc Natl Acad Sci U S A*. 1991;88:10480–10484.
- Rapoport RM, Draznin MB, Murad F. Endothelium-dependent relaxation in rat aorta may be mediated through cyclic GMP-dependent protein phosphorylation. *Nature*. 1983;306:174–176.
- Wilcox JN, Subramanian RR, Sundell CL, Tracey WR, Pollock JS, Harrison DG, Marsden PA. Expression of multiple isoforms of nitric oxide synthase in normal and atherosclerotic vessels. *Arterioscler Thromb Vasc Biol*. 1997;17:2479–2488.
- Buttery LD, Springall DR, Chester AH, Evans TJ, Standfield EN, Parums DV, Yacoub MH, Polak JM. Inducible nitric oxide synthase is present within human atherosclerotic lesions and promotes the formation and activity of peroxynitrite. *Lab Invest*. 1996;75:77–85.
- Behr D, Rupin A, Fabiani JN, Verbeuren TJ. Distribution and prevalence of inducible nitric oxide synthase in atherosclerotic vessels of long-term cholesterol-fed rabbits. *Atherosclerosis*. 1999;142:335–344.
- Kuhlencordt PJ, Chen J, Han F, Astern J, Huang PL. Genetic deficiency of inducible nitric oxide synthase reduces atherosclerosis and lowers plasma lipid peroxides in apolipoprotein E- knockout mice. *Circulation*. 2001;103:3099–3104.
- Tzeng E, Billiar TR, Robbins PD, Loftus M, Stuehr DJ. Expression of human inducible nitric oxide synthase in a tetrahydrobiopterin (H4B)-deficient cell line: H4B promotes assembly of enzyme subunits into an active dimer. *Proc Natl Acad Sci U S A*. 1995;92:11771–11775.
- Cho HJ, Martin E, Xie QW, Sassa S, Nathan C. Inducible nitric oxide synthase: identification of amino acid residues essential for dimerization and binding of tetrahydrobiopterin. *Proc Natl Acad Sci U S A*. 1995;92:11514–11518.
- Harrison DG. Cellular and molecular mechanisms of endothelial cell dysfunction. *J Clin Invest*. 1997;100:2153–2157.
- De Caterina R, Libby P, Peng HB, Thannickal VJ, Rajavashisth TB, Gimbrone MA, Jr, Shin WS, Liao JK. Nitric oxide decreases cytokine-induced endothelial activation. Nitric oxide selectively reduces endothelial expression of adhesion molecules and proinflammatory cytokines. *J Clin Invest*. 1995;96:60–68.
- Khan BV, Harrison DG, Olbrych MT, Alexander RW, Medford RF. Nitric oxide regulates cell adhesion molecule 1 and redox-sensitive transcriptional events in human vascular endothelial cells. *Proc Natl Acad Sci U S A*. 1996;93:9114–9119.
- Nunes GL, Robinson K, Kalynych A, King SB III, Sgoutas DS, Berk BC. Vitamins C and E inhibit O₂⁻ production in the pig coronary artery. *Circulation*. 1997;96:3593–3601.
- Pratico D, Tangirala RK, Rader DJ, Rokach J, FitzGerald GA. Vitamin E suppresses isoprostane generation in vivo and reduces atherosclerosis in ApoE-deficient mice. *Nat Med*. 1998;4:1189–1192.
- Bauersachs J, Fleming I, Fraccarollo D, Busse R, Ertl G. Prevention of endothelial dysfunction in heart failure by vitamin E: attenuation of vascular superoxide anion formation and increase in soluble guanylyl cyclase expression. *Cardiovasc Res*. 2001;51:344–350.
- Chen X, Touyz RM, Park JB, Schiffrin EL. Antioxidant effects of vitamins C and E are associated with altered activation of vascular NADPH oxidase and superoxide dismutase in stroke-prone SHR. *Hypertension*. 2001;38:606–611.
- Keaney JF Jr, Gaziano JM, Xu A, Frei B, Curran-Celentano J, Shwaery GT, Loscalzo J, Vita JA. Low-dose alpha-tocopherol improves and high-dose alpha-tocopherol worsens endothelial vasodilator function in cholesterol-fed rabbits. *J Clin Invest*. 1994;93:844–851.
- Böger RH, Bode-Böger SM, Phivthong-Ngam L, Brandes RP, Schwedhelm E, Mügge A, Bohme M, Tsikas D, Frölich JC. Dietary L-arginine and α -tocopherol reduce vascular oxidative stress and preserve endothelial function in hypercholesterolemic rabbits via different mechanisms. *Atherosclerosis*. 1998;141:31–43.
- Ting HH, Timimi FK, Haley EA, Roddy MA, Ganz P, Creager MA. Vitamin C improves endothelium-dependent vasodilation in forearm resistance vessels of humans with hypercholesterolemia. *Circulation*. 1997;95:2617–2622.
- Heitzer T, Just H, Münzel T. Antioxidant vitamin C improves endothelial dysfunction in chronic smokers. *Circulation*. 1996;94:6–9.
- Taddei S, Virdis A, Ghiadoni L, Magagna A, Salvetti A. Vitamin C improves endothelium-dependent vasodilation by restoring nitric oxide activity in essential hypertension. *Circulation*. 1998;97:2222–2229.

26. Levine GN, Frei B, Koulouris SN, Gerhard MD, Keaney JF, Jr, Vita JA. Ascorbic acid reverses endothelial vasomotor dysfunction in patients with coronary artery disease. *Circulation*. 1996;93:1107–1113.
27. Gokce N, Keaney JF Jr, Frei B, Holbrook M, Olesiak M, Zachariah BJ, Leeuwenburgh C, Heinecke JW, Vita JA. Long-term ascorbic acid administration reverses endothelial vasomotor dysfunction in patients with coronary artery disease. *Circulation*. 1999;99:3234–3240.
28. Huang A, Vita JA, Venema RC, Keaney JF, Jr. Ascorbic acid enhances endothelial nitric oxide synthase activity by increasing intracellular tetrahydrobiopterin. *J Biol Chem*. 2000;275:17399–17406.
29. Baker TA, Milstien S, Katusic ZS. Effect of vitamin C on the availability of tetrahydrobiopterin in human endothelial cells. *J Cardiovasc Pharmacol*. 2001;37:333–338.
30. Heller R, Unbehauen A, Schellenberg B, Mayer B, Werner-Felmayer G, Werner ER. L-ascorbic acid potentiates endothelial nitric oxide synthesis via a chemical stabilization of tetrahydrobiopterin. *J Biol Chem*. 2001;276:40–47.
31. Breslow JL. Mouse models of atherosclerosis. *Science*. 1996;272:685–688.
32. Plump AS, Smith JD, Hayek T, Aalto-Setälä K, Walsh A, Verstuyft JG, Rubin EM, Breslow JL. Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. *Cell*. 1992;71:343–353.
33. Tsao CS, Leung PY, Young M. Effect of dietary ascorbic acid intake on tissue vitamin C in mice. *J Nutr*. 1987;117:291–297.
34. Sheehan DC, Hrapchak BB. *Theory and Practice of Histotechnology*, 2nd ed. Columbus, Ohio: Battelle Press; 1987.
35. d'Uscio LV, Baker TA, Mantilla CB, Smith L, Weiler D, Sieck GC, Katusic ZS. Mechanism of endothelial dysfunction in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol*. 2001;21:1017–1022.
36. Fukushima T, Nixon JC. Analysis of reduced forms of biopterin in biological tissues and fluids. *Anal Biochem*. 1980;102:176–188.
37. Tsutsui M, Milstien S, Katusic ZS. Effect of tetrahydrobiopterin on endothelial function in canine middle cerebral arteries. *Circ Res*. 1996;79:336–342.
38. Milstien S, Katusic Z. Oxidation of tetrahydrobiopterin by peroxynitrite: implications for vascular endothelial function. *Biochem Biophys Res Commun*. 1999;263:681–684.
39. Yamashita T, Kawashima S, Ohashi Y, Ozaki M, Rikitake Y, Inoue N, Hirata K, Akita H, Yokoyama M. Mechanisms of reduced nitric oxide/cGMP-mediated vasorelaxation in transgenic mice overexpressing endothelial nitric oxide synthase. *Hypertension*. 2000;36:97–102.
40. Gunneth CA, Lund DD, Chu Y, Brooks RM, 2nd, Faraci FM, Heistad DD. NO-dependent vasorelaxation is impaired after gene transfer of inducible NO-synthase. *Arterioscler Thromb Vasc Biol*. 2001;21:1281–1287.
41. Weber M, Lauer N, Mulsch A, Kojda G. The effect of peroxynitrite on the catalytic activity of soluble guanylyl cyclase. *Free Radic Biol Med*. 2001;31:1360–1367.
42. Kinoshita H, Milstien S, Wambi C, Katusic ZS. Inhibition of tetrahydrobiopterin biosynthesis impairs endothelium-dependent relaxations in canine basilar artery. *Am J Physiol*. 1997;273:H718–H724.
43. Werner-Felmayer G, Werner ER, Fuchs D, Hausen A, Reibnegger G, Schmidt K, Weiss G, Wachter H. Pteridine biosynthesis in human endothelial cells. Impact on nitric oxide-mediated formation of cyclic GMP. *J Biol Chem*. 1993;268:1842–1846.
44. Rosenkranz-Weiss P, Sessa WC, Milstien S, Kaufman S, Watson CA, Poher JS. Regulation of nitric oxide synthesis by proinflammatory cytokines in human umbilical vein endothelial cells. Elevations in tetrahydrobiopterin levels enhance endothelial nitric oxide synthase specific activity. *J Clin Invest*. 1994;93:2236–2243.
45. Simmons WW, Ungureanu-Longrois D, Smith GK, Smith TW, Kelly RA. Glucocorticoids regulate inducible nitric oxide synthase by inhibiting tetrahydrobiopterin synthesis and L-arginine transport. *J Biol Chem*. 1996;271:23928–23937.
46. Katusic ZS, Stelter A, Milstien S. Cytokines stimulate GTP cyclohydrolase I gene expression in cultured human umbilical vein endothelial cells. *Arterioscler Thromb Vasc Biol*. 1998;18:27–32.
47. Tatzber F, Rabl H, Koriska K, Erhart U, Puhl H, Waeg G, Krebs A, Esterbauer H. Elevated serum neopterin levels in atherosclerosis. *Atherosclerosis*. 1991;89:203–208.
48. Schumacher M, Halwachs G, Tatzber F, Fruhwald FM, Zweiker R, Watzinger N, Eber B, Wilders-Truschnig M, Esterbauer H, Klein W. Increased neopterin in patients with chronic and acute coronary syndromes. *J Am Coll Cardiol*. 1997;30:703–707.
49. Laursen JB, Somers M, Kurz S, McCann L, Warnholtz A, Freeman BA, Tarpey M, Fukui T, Harrison DG. Endothelial regulation of vasomotion in apoE-deficient mice: implications for interactions between peroxynitrite and tetrahydrobiopterin. *Circulation*. 2001;103:1282–1288.
50. Zou MH, Shi C, Cohen RA. Oxidation of the zinc-thiolate complex and uncoupling of endothelial nitric oxide synthase by peroxynitrite. *J Clin Invest*. 2002;109:817–826.
51. Vasquez-Vivar J, Whittsett J, Martasek P, Hogg N, Kalyanaraman B. Reaction of tetrahydrobiopterin with superoxide: EPR-kinetic analysis and characterization of the pteridine radical. *Free Radic Biol Med*. 2001;31:975–985.
52. Patel KB, Stratford MR, Wardman P, Everett SA. Oxidation of tetrahydrobiopterin by biological radicals and scavenging of the trihydrobiopterin radical by ascorbate. *Free Radic Biol Med*. 2002;32:203–211.
53. Hofmann H, Schmidt HH. Thiol dependence of nitric oxide synthase. *Biochemistry*. 1995;34:13443–13452.
54. Kurl S, Tuomainen TP, Laukkanen JA, Nyyssonen K, Lakka T, Sivenius J, Salonen JT. Plasma vitamin C modifies the association between hypertension and risk of stroke. *Stroke*. 2002;33:1568–1573.
55. Ness AR, Khaw KT, Bingham S, Day NE. Vitamin C status and blood pressure. *J Hypertens*. 1996;14:503–508.
56. Weber P, Bendich A, Schalch W. Vitamin C and human health—a review of recent data relevant to human requirements. *Int J Vitam Nutr Res*. 1996;66:19–30.
57. Langlois M, Duprez D, Delanghe J, De Buyzere M, Clement DL. Serum vitamin C concentration is low in peripheral arterial disease and is associated with inflammation and severity of atherosclerosis. *Circulation*. 2001;103:1863–1868.
58. Ek A, Strom K, Cotgreave IA. The uptake of ascorbic acid into human umbilical vein endothelial cells and its effect on oxidant insult. *Biochem Pharmacol*. 1995;50:1339–1346.
59. Jackson TS, Xu A, Vita JA, Keaney JF, Jr. Ascorbate prevents the interaction of superoxide and nitric oxide only at very high physiological concentrations. *Circ Res*. 1998;83:916–922.
60. Katusic ZS. Vascular endothelial dysfunction: does tetrahydrobiopterin play a role? *Am J Physiol Heart Circ Physiol*. 2001;281:H981–H986.