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Potential Antiatherogenic Mechanisms of Ascorbate (Vitamin C) and  $\alpha$ -Tocopherol (Vitamin E)

Crosstalk Between Nitric Oxide and Lipid Oxidation Systems: Implications for Vascular Disease

Oxygen Radicals and Endothelial Dysfunction

Vascular Oxygen Species Generation

David G. Harrison, Guest Editor

## Potential Antiatherogenic Mechanisms of Ascorbate (Vitamin C) and $\alpha$ -Tocopherol (Vitamin E)

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**Abstract**—The premise that oxidative stress, among several other factors, plays an important role in atherogenesis implies that the development and progression of atherosclerosis can be inhibited by antioxidants. In this minireview we discuss several mechanisms by which the antioxidants ascorbate (vitamin C) and  $\alpha$ -tocopherol (vitamin E) may protect against atherosclerosis. These mechanisms include inhibition of LDL oxidation and inhibition of leukocyte adhesion to the endothelium and vascular endothelial dysfunction. Overall, ascorbate appears to be more effective than  $\alpha$ -tocopherol in mitigating these pathophysiological processes, most likely as a result of its abilities to effectively scavenge a wide range of reactive oxygen and nitrogen species and to regenerate  $\alpha$ -tocopherol, and possibly tetrahydrobiopterin, from its radical species. In contrast,  $\alpha$ -tocopherol can act either as an antioxidant or a pro-oxidant to inhibit or facilitate, respectively, lipid peroxidation in LDL. However, this pro-oxidant activity of  $\alpha$ -tocopherol is prevented by ascorbate acting as a coantioxidant. Therefore, an optimum vitamin C intake or body status may help protect against atherosclerosis and its clinical sequelae, whereas vitamin E may only be effective in combination with vitamin C. (*Circ Res.* 2000;87:349-354.)

**Key Words:** ascorbic acid ■ atherosclerosis ■ endothelial cells ■ low-density lipoprotein ■ vitamin E

Oxidative stress is thought to play an important role in atherosclerotic vascular disease.<sup>1</sup> Thus, dietary antioxidants such as ascorbate (vitamin C) and  $\alpha$ -tocopherol (the chemically and biologically most active form of vitamin E) can protect against the development and progression of atherosclerosis in experimental models.<sup>1</sup> Numerous observational studies have shown an inverse association between antioxidant intake or body status and the risk of cardiovascular diseases.<sup>2</sup> However, several clinical trials, such as the recent GISSI and HOPE trials, have found no benefits of vitamin E supplementation on cardiovascular disease risk.<sup>3</sup> A major caveat with observational studies is that they can only

show associations and not causal relationships. Clinical trials have a number of limitations, too, such as a relatively short period of antioxidant treatment with a single dose only, and the fact that antioxidant treatment of patients with advanced disease (secondary prevention) may not provide information relevant to disease prevention in healthy individuals (primary prevention). For example, both the GISSI and HOPE trials were secondary prevention trials in which >75% of all participants were treated with aspirin or other antiplatelet agents, and many participants also received  $\beta$ -blockers, lipid-lowering agents, and calcium channel blockers.<sup>3</sup> It is doubtful whether vitamin E can exert beneficial effects above

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and beyond these standard therapies. In addition, as explained in the current article, vitamin E supplements alone may not be beneficial but may have to be coadministered with vitamin C to effectively lower oxidative stress. For further detailed discussions of the observational and clinical trial data regarding vitamins C and E, see References 4 and 3, respectively.

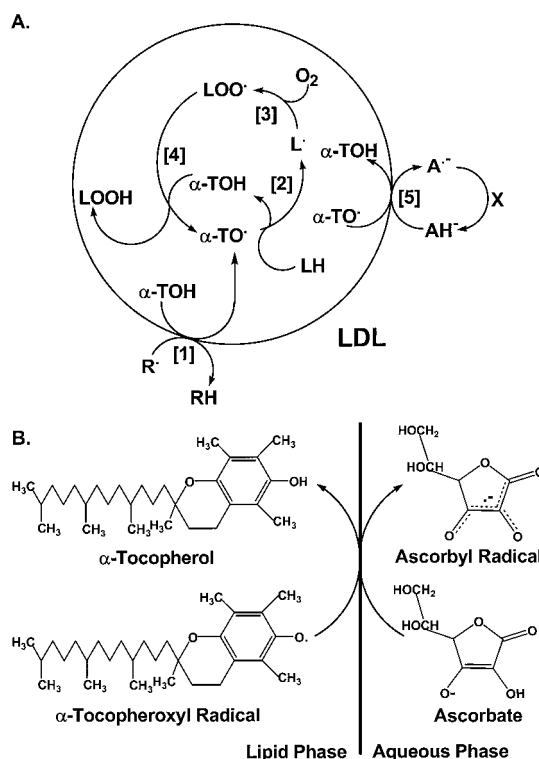
Understanding the specific mechanisms by which ascorbate and  $\alpha$ -tocopherol exert their protective effects will help elucidate their potential roles in atherogenesis and may ultimately lead to successful preventive or therapeutic regimens. A number of antiatherogenic mechanisms have been proposed, and of these several will be discussed in this minireview. They include inhibition of LDL oxidation by LDL-associated  $\alpha$ -tocopherol or extracellular ascorbate and inhibition of leukocyte–endothelial cell interactions and vascular dysfunction by both extracellular and intracellular ascorbate and  $\alpha$ -tocopherol.

### LDL Oxidation

Oxidatively modified LDL has been implicated in the pathogenesis of atherosclerosis.<sup>1,2</sup> Although the mode of LDL oxidation in vivo is incompletely understood, the mechanisms of LDL oxidation in vitro have been studied extensively.<sup>1</sup> Modification of the protein moiety of LDL (apolipoprotein B-100), either directly by leukocyte-derived oxidants such as hypochlorous acid<sup>5</sup> or indirectly by lipid hydroperoxide breakdown products such as 4-hydroxynonenal and malondialdehyde,<sup>6</sup> results in a form of LDL that is internalized by macrophages via the scavenger receptor pathway leading to foam cell formation.<sup>1,7</sup> Although redox-active transition metal ions seem to play a pivotal role in cell-mediated LDL oxidation,<sup>1,7</sup> the presence of free copper or iron ions in vivo is doubtful. Various metal ion-independent mechanisms of LDL oxidation have been proposed, such as reactive nitrogen and chlorine species.<sup>5</sup> Furthermore, there is convincing evidence that in vitro lipid peroxidation in LDL is initiated by  $\alpha$ -tocopheroxyl radicals formed in the lipoprotein on attack by free radicals or other reactive species<sup>8,9</sup> (Figure 1A). Thus,  $\alpha$ -tocopherol can act as a pro-oxidant, rather than an antioxidant, in LDL incubated in vitro<sup>8,9</sup> (see below).

### Ascorbate and LDL Oxidation

Human plasma and other extracellular fluids contain numerous water-soluble antioxidants, including ascorbate, urate, bilirubin, and various thiol compounds.<sup>10</sup> Experimental data on the effects of vitamin C supplementation of human subjects on ex vivo LDL oxidation are sparse, mainly because ascorbate is removed from LDL during isolation from plasma.<sup>11</sup> However, there is convincing evidence from in vitro studies that physiological concentrations of ascorbate strongly inhibit LDL oxidation by vascular cells and neutrophils,<sup>12,13</sup> as well as in cell-free systems.<sup>14,15</sup> Ascorbate prevents oxidative modification of LDL primarily by scavenging free radicals and other reactive species in the aqueous milieu.<sup>10</sup> Thus, direct and rapid trapping of these aqueous reactive species by ascorbate prevents them from interacting with and oxidizing LDL. Ascorbyl radicals formed in this process may be reduced back to ascorbate by dismutation, chemical reduction (eg, by glutathione), or enzymatic reduc-



**Figure 1.** A, Tocopherol-mediated peroxidation in LDL in vitro. Tocopherol-mediated peroxidation is initiated by reaction [1], also called the phase-transfer reaction of  $\alpha$ -tocopherol ( $\alpha$ -TOH). Lipid peroxidation initiation (reaction [2]), followed by the propagation reactions [3] and [4], reflect the chain-transfer activity of  $\alpha$ -TOH. Ascorbate ( $AH^{\cdot}$ ) inhibits tocopherol-mediated peroxidation by reacting with the  $\alpha$ -tocopheroxyl radical ( $\alpha$ -TO $\cdot$ ) to regenerate  $\alpha$ -TOH (reaction [5]).  $AH^{\cdot}$  may be regenerated (X) from the ascorbyl radical ( $A^{\cdot}$ ) by dismutation, chemical reduction, or enzymatic reduction (see text). LH indicates lipid molecule containing a polyunsaturated fatty acyl side chain; L $\cdot$ , carbon-centered lipid radical; LOO $\cdot$ , lipid peroxy radical; LOOH, lipid hydroperoxide; R $\cdot$ , radical oxidant; and RH, reduced form of the radical oxidant. (Modified from *Free Radical Biology & Medicine*, Vol 22, J. Neuzil, S.R. Thomas, R. Stocker, Requirement for, promotion, or inhibition by  $\alpha$ -tocopherol of radical-induced initiation of plasma lipoprotein lipid peroxidation, pp 57–71, copyright 1997, with permission from Elsevier Science).<sup>9</sup> B, Interaction of ascorbate with the  $\alpha$ -tocopheroxyl radical. Standard reduction potentials for  $AH^{\cdot}$ ,  $A^{\cdot}/H^+$  and  $\alpha$ -TOH,  $\alpha$ -TO $\cdot/H^+$  are  $-0.28$  and  $-0.50$  V, respectively.<sup>64</sup>

tion (eg, by thioredoxin reductase).<sup>16</sup> Dismutation also produces dehydroascorbic acid, which in turn can be reduced back to ascorbate by glutathione, thioredoxin reductase, and glutaredoxin.<sup>17</sup> Ascorbate can also prevent the pro-oxidant activity of  $\alpha$ -tocopherol by reducing the  $\alpha$ -tocopheroxyl radical to  $\alpha$ -tocopherol, thereby acting as a “coantioxidant” and inhibiting LDL oxidation<sup>9,13</sup> (Figures 1A and 1B).

### $\alpha$ -Tocopherol and LDL Oxidation

Human LDL contains various lipid-soluble antioxidants, including  $\alpha$ -tocopherol,  $\gamma$ -tocopherol, ubiquinol-10, and several carotenoids and oxycarotenoids.<sup>13</sup>  $\alpha$ -Tocopherol, the most abundant antioxidant in LDL,<sup>13</sup> can act as a chain-breaking antioxidant by scavenging highly reactive lipid peroxy and alkoxy radicals, which otherwise would propagate the chain reaction of lipid peroxidation. Esterbauer et al<sup>6</sup>

reported that  $\alpha$ -tocopherol-depleted LDL is able to undergo rapid lipid peroxidation, whereas LDL isolated from  $\alpha$ -tocopherol-supplemented subjects exhibits increased resistance to ex vivo copper-induced oxidation.<sup>18</sup> However, various investigators have since reported that the resistance of LDL to oxidation does not correlate with its vitamin E content<sup>2,9</sup> and that ubiquinol-10, not  $\alpha$ -tocopherol, forms the first line of antioxidant defense in human LDL.<sup>13</sup> In particular, Bowry and Stocker<sup>8</sup> and Neuzil et al<sup>9</sup> reported that  $\alpha$ -tocopherol can act as a pro-oxidant in LDL via  $\alpha$ -tocopheroxyl radical-mediated formation of lipid radicals (Figure 1A). Accordingly, in vitro and in vivo enrichment of LDL with  $\alpha$ -tocopherol accelerates rather than inhibits the initial stages of LDL oxidation.<sup>8</sup> These results do not refute a role for  $\alpha$ -tocopherol as an antioxidant in vivo, given that coantioxidants, such as ascorbate<sup>19</sup> and ubiquinol-10,<sup>13</sup> are present in the vascular environment and can convert  $\alpha$ -tocopherol from a pro-oxidant into an antioxidant.<sup>9,13</sup>

### Cell Adhesion

Adhesion of leukocytes to the endothelium is an important initiating step in atherogenesis.<sup>20–22</sup> Various studies have shown that monocytes bind selectively to aortic prelesion areas and atherosclerotic lesions,<sup>20</sup> which also exhibit increased expression of adhesion molecules compared with normal tissue.<sup>21</sup> Cultured endothelial cells exposed to inflammatory cytokines or oxidized LDL exhibit enhanced expression of cell adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin.<sup>22</sup> These adhesion molecules interact with specific ligands expressed on the surface of leukocytes, such as the  $\beta_1$  and  $\beta_2$  integrins, and mediate leukocyte rolling, firm attachment to the endothelium, and subsequent migration into the subendothelial space.<sup>22</sup>

### Ascorbate and Cell Adhesion

Two recent human studies have investigated the role of ascorbate in inhibiting cell-cell adhesion.<sup>23,24</sup> Smokers have decreased plasma levels of ascorbate, and monocytes isolated from smokers exhibit increased adhesion to cultured endothelial cells compared with monocytes isolated from non-smokers.<sup>23,24</sup> Supplementation of smokers with 2 g/day of vitamin C for 10 days elevated plasma ascorbate levels almost 2-fold and significantly reduced monocyte adhesion to cultured endothelial cells.<sup>23</sup> This finding indicates that up-regulation of ligands on monocytes is inhibited by ascorbate.<sup>23</sup> In another study, however, supplementation of smokers with 2 g of vitamin C 2 hours before isolation of monocytes had no effect on ex vivo monocyte-endothelial cell adhesion or endothelial ICAM-1 surface expression, despite a >3-fold increase in serum ascorbate levels.<sup>24</sup> The supplementation period in this study may have been too short to affect intracellular ascorbate levels.

Several in vivo studies using intravital microscopy in hamsters have demonstrated an important role of ascorbate in inhibiting leukocyte-endothelial cell interactions induced by cigarette smoke<sup>25,26</sup> or oxidized LDL,<sup>27</sup> likely by antioxidant mechanisms. Lehr et al<sup>26</sup> showed that the induction of leukocyte adhesion to the vascular wall elicited by cigarette

smoke is due to the formation of oxidatively modified lipids with platelet-activating factor-like activity. Administration of ascorbate prevented the accumulation of these platelet-activating factor-like lipids and the subsequent leukocyte-endothelial cell interactions.<sup>26</sup>

### $\alpha$ -Tocopherol and Cell Adhesion

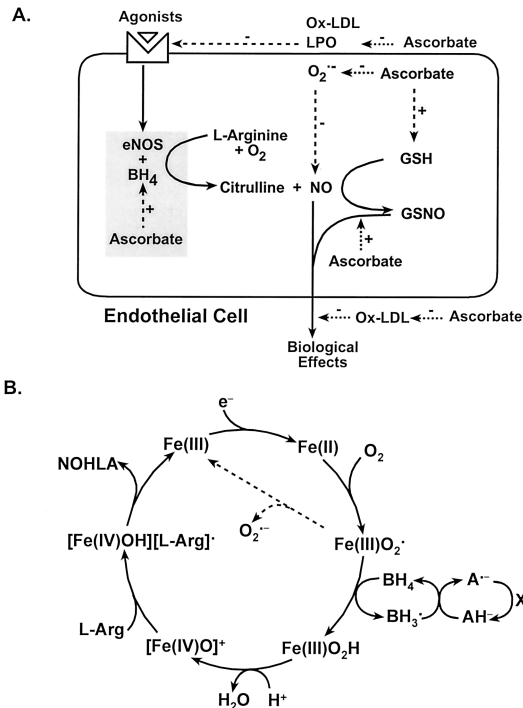
Cell culture studies have shown that pretreatment of endothelial cells with  $\alpha$ -tocopherol inhibits cytokine or oxidized LDL-induced expression of ICAM-1, VCAM-1, or E-selectin and decreases adhesion of monocytes to these cells.<sup>28–31</sup> Interestingly, Cominacini et al<sup>30</sup> found that both LDL-associated and cellular  $\alpha$ -tocopherol are able to inhibit upregulation of ICAM-1 and VCAM-1 by endothelial cells exposed to oxidized LDL.  $\alpha$ -Tocopherol also decreased stimulus-induced expression of  $\beta_1$  and  $\beta_2$  integrins on leukocytes and adhesion of these cells to cultured endothelial cells.<sup>28,32,33</sup> Ex vivo studies in humans have shown an inverse correlation between serum  $\alpha$ -tocopherol levels and  $\beta_1$  integrin expression on monocytes,<sup>32</sup> as well as decreased ex vivo monocyte-endothelial cell adhesion<sup>34</sup> after supplementation with  $\alpha$ -tocopherol. Another study, however, showed no effect on monocyte adhesiveness after supplementation of hypercholesterolemic patients with  $\alpha$ -tocopherol.<sup>35</sup>

$\alpha$ -Tocopherol could inhibit the expression of adhesion molecules and subsequent cell-cell interactions either by directly scavenging reactive oxygen species (ROS) or by inhibiting protein kinase C (PKC) activation and associated ROS production.<sup>36</sup> Although one study found no change in PKC activity in endothelial cells treated with  $\alpha$ -tocopherol, despite decreased E-selectin expression,<sup>29</sup> other studies showed decreased PKC activity paralleled by decreased ROS production in leukocytes treated with  $\alpha$ -tocopherol.<sup>32,34</sup>

Despite convincing evidence that  $\alpha$ -tocopherol decreases cell-cell adhesion in vitro and ex vivo, evidence is completely lacking in vivo.<sup>25,27,37</sup> For example,  $\alpha$ -tocopherol supplementation of hamsters had no effect on leukocyte-endothelial cell interactions induced by cigarette smoke<sup>25</sup> or oxidized LDL.<sup>27</sup> More studies are needed to determine whether  $\alpha$ -tocopherol and ascorbate exert a consistent inhibitory effect on in vivo leukocyte-endothelial cell interactions and to further elucidate the underlying mechanisms.

### Endothelial NO Synthesis

Endothelium-derived NO (EDNO) is a pivotal molecule in the regulation of vascular tone and homeostasis.<sup>38</sup> In addition to stimulating vascular smooth muscle cell relaxation and vasodilation, EDNO exerts a number of potent antiatherogenic effects, including inhibition of smooth muscle cell proliferation, platelet aggregation, and leukocyte-endothelial cell interactions.<sup>38</sup> EDNO is synthesized from L-arginine through the action of constitutive and inducible isoforms of the NADPH-dependent enzyme NO synthase (NOS, Figure 2A).<sup>39</sup> The enzyme requires a number of cofactors, including flavin adenine dinucleotide, flavin mononucleotide, tetrahydrobiopterin, and possibly thiols.<sup>39</sup> Endothelial vasodilator dysfunction has been observed in patients with coronary artery disease or subjects with coronary risk factors.<sup>40</sup> Most of these conditions are associated with increased oxidative



**Figure 2.** A, Potential mechanisms by which ascorbate enhances the synthesis and preserves the biological activity of EDNO. Ascorbate scavenges superoxide radicals ( $O_2^{\cdot -}$ ) and prevents plasma membrane lipid peroxidation (LPO), which otherwise would decrease NO levels either by direct reaction of  $O_2^{\cdot -}$  with NO or by interruption of agonist-induced NO synthesis. Ascorbate also inhibits the formation of oxidized LDL (Ox-LDL), which can decrease the synthesis and biological activity of NO. Ascorbate has a number of other functions, as follows: sparing intracellular glutathione (GSH), which in turn can stabilize NO through the formation of biologically active S-nitrosoglutathione (GSNO); release of NO from S-nitrosothiols; and preservation of cofactors of endothelial NOS (eNOS), in particular tetrahydrobiopterin ( $BH_4$ ). Solid arrows indicate reactions; dashed arrows, effects;  $-$ , inhibition; and  $+$ , stimulation. B, Proposed interaction of ascorbate and tetrahydrobiopterin in the first half-reaction of the NOS cycle. Ascorbate ( $AH^-$ ) reduces the trihydrobiopterin radical ( $BH_3^{\cdot -}$ ) to tetrahydrobiopterin ( $BH_4$ ), thus inhibiting superoxide production by the uncoupled reaction (dashed line) and stimulating the conversion of L-arginine (L-Arg) to N-hydroxy-L-arginine (NOHLA). Regeneration of  $AH^-$  from the ascorbyl radical ( $A^{\cdot -}$ ), indicated by X, is explained in the legend to Figure 1. (Modified with permission from Bec N, Gorren AC, Voelker C, Mayer B, Lange R. Reaction of neuronal nitric-oxide synthase with oxygen at low temperature: evidence for reductive activation of the oxy-ferrous complex by tetrahydrobiopterin. *J Biol Chem*. 1998;273:13502–13508<sup>65</sup>).

stress, particularly increased production of superoxide radicals, which can inactivate EDNO.<sup>41</sup> In addition, oxidized LDL has been shown to inhibit the synthesis of EDNO or attenuate its biological activity.<sup>42</sup>

### Ascorbate and EDNO

Numerous clinical studies have consistently demonstrated beneficial effects of vitamin C treatment on endothelium-dependent vasodilation in individuals with coronary artery disease or coronary risk factors (reviewed in Reference 4). There are a number of potential mechanisms underlying the salubrious effects of ascorbate on endothelial function (Figure 2A). First, ascorbate may be decreasing the levels of

superoxide radicals and oxidized LDL,<sup>43,44</sup> both of which react with and inactivate NO.<sup>41,42</sup> Because of the facile reaction between superoxide and NO radicals, relatively high concentrations of ascorbate ( $\approx 10$  mmol/L) are required to effectively inhibit the reaction of NO with superoxide.<sup>44</sup> Such concentrations are potentially achievable in plasma by intra-arterial infusion<sup>4</sup> or in the cytoplasm as a result of cellular uptake of ascorbate.<sup>45–47</sup>

Ascorbate may indirectly enhance endothelium-dependent vasodilation by sparing intracellular thiols,<sup>48</sup> which in turn stabilize EDNO through the formation of biologically active S-nitrosothiols<sup>49</sup> (Figure 2A). Reducing agents such as ascorbate have also been implicated in the rapid release of NO from S-nitrosothiols.<sup>50</sup> Finally, Heller et al<sup>46</sup> and, more recently, Huang et al<sup>47</sup> have shown that physiological concentrations of ascorbate increase the synthesis and biological activity of NO in cultured endothelial cells by increasing intracellular tetrahydrobiopterin (Figure 2B). Thus, a very likely mechanism by which intracellular ascorbate stimulates NOS activity is regeneration of tetrahydrobiopterin from the trihydrobiopterin radical (Figure 2B). Such a mechanism of action of ascorbate would also prevent NOS from leaking superoxide radicals (Figure 2B).

### $\alpha$ -Tocopherol and EDNO

Animal studies have provided consistent evidence for a beneficial effect of  $\alpha$ -tocopherol on vasodilation, as well as insight into underlying mechanisms. Supplementation of cholesterol-fed rabbits with  $\alpha$ -tocopherol increased both the resistance of LDL to oxidation and agonist-induced relaxation of thoracic aortas, whereas supplementation with  $\beta$ -carotene had no effect on LDL oxidizability yet did enhance agonist-induced vasodilation.<sup>51</sup> These results suggest that  $\beta$ -carotene and  $\alpha$ -tocopherol act by increasing vascular antioxidant status rather than LDL antioxidant status. In addition,  $\alpha$ -tocopherol may act by non-antioxidant mechanisms. For example, Keaney et al<sup>52</sup> proposed that  $\alpha$ -tocopherol acts in the vascular wall by inhibiting PKC activation by oxidized LDL, hence inhibiting PKC-mediated phosphorylation of endothelial cell muscarinic receptors and enhancing agonist-induced NOS activation. In contrast, supplementation of cholesterol-fed rabbits with supraphysiological amounts of  $\alpha$ -tocopherol profoundly impaired agonist-induced aortic relaxation and also increased the extent of intimal proliferation.<sup>52a</sup> The mechanisms for these adverse effects of high doses of  $\alpha$ -tocopherol are unclear, but they may be related to the pro-oxidant activity of  $\alpha$ -tocopherol in LDL (see Figure 1A).

A number of clinical studies have shown that  $\alpha$ -tocopherol increases endothelium-dependent vasodilation in individuals with coronary risk factors.<sup>37,53–56</sup> Two of these studies also showed a reduction in markers of lipid oxidation.<sup>53,54</sup> Other human studies, however, did not find an effect of  $\alpha$ -tocopherol supplementation on endothelium-dependent vasodilation<sup>57–59</sup> or on lipid peroxidation.<sup>57,59</sup>

Combinations of antioxidants may be of particular benefit because of the possible synergistic interaction between ascorbate and  $\alpha$ -tocopherol (see Figure 1B). Of 4 studies in which individuals with coronary risk factors were supplemented with  $\alpha$ -tocopherol in combination with ascorbate,<sup>60–63</sup> only 1

showed no effect on agonist-induced vasodilation, despite a reduction in the susceptibility of LDL to ex vivo oxidation.<sup>63</sup>

Taken together, these data indicate that ascorbate, alone or in combination with  $\alpha$ -tocopherol, enhances the synthesis and biological activity of EDNO through several antioxidant mechanisms, in particular regeneration of tetrahydrobiopterin.  $\alpha$ -Tocopherol may improve EDNO levels via the inhibition of PKC activity; however, in humans there are insufficient data to conclude that long-term treatment with  $\alpha$ -tocopherol alone is beneficial.

### Summary

The formation and activation of atherosclerotic lesions is a multifaceted process involving many determinants, including oxidative modification of LDL. Oxidized LDL causes foam cell formation, leukocyte adhesion to the endothelium, cytotoxicity, and vascular endothelial dysfunction leading to impaired EDNO synthesis and biological activity.<sup>1,2,7</sup> Current evidence strongly suggests, although it does not prove, that ascorbate and  $\alpha$ -tocopherol protect against atherogenesis by inhibiting LDL oxidation, by impairing the production of ROS by vascular cells, and by limiting the cellular responses to oxidized LDL, in particular adhesion molecule expression and EDNO synthesis. Some of the beneficial effects of  $\alpha$ -tocopherol may be attenuated by the pro-oxidant effects on lipid peroxidation in LDL. However, this pro-oxidant activity of LDL-associated  $\alpha$ -tocopherol is counteracted by ascorbate present in plasma and the arterial wall.<sup>10,19</sup> Although the current evidence is promising, more mechanistic and human in vivo studies are needed to determine whether optimizing the dietary intake or body status of vitamins C and E can help decrease atherosclerotic vascular disease and its clinical sequelae.

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