On the Role of Vitamin C and Other Antioxidants in Atherogenesis and Vascular Dysfunction (44444)

BALZ FREI[1]

Linus Pauling Institute, Oregon State University, Corvallis, Oregon 97331–6512

Abstract. **Oxidative stress has been implicated as an important etiologic factor in atherosclerosis and vascular dysfunction. Antioxidants may inhibit atherogenesis and improve vascular function by two different mechanisms. First, lipid-soluble antioxidants present in low-density lipoprotein (LDL), including α-tocopherol, and water-soluble antioxidants present in the extracellular fluid of the arterial wall, including ascorbic acid (vitamin C), inhibit LDL oxidation through an LDL-specific antioxidant action. Second, antioxidants present in the cells of the vascular wall decrease cellular production and release of reactive oxygen species (ROS), inhibit endothelial activation (i.e., expression of adhesion molecules and monocyte chemoattractants), and improve the biologic activity of endothelium-derived nitric oxide (EDNO) through a cell- or tissue-specific antioxidant action. α-Tocopherol and a number of thiol antioxidants have been shown to decrease adhesion molecule expression and monocyte-endothelial interactions. Vitamin C has been demonstrated to potentiate EDNO activity and normalize vascular function in patients with coronary artery disease and associated risk factors, including hypercholesterolemia, hyperhomocysteinemia, hypertension, diabetes, and smoking.** [P.S.E.B.M. 1999, Vol 222]

The Pathogenesis of Atherosclerosis

Atherosclerosis is a chronic disease of large and medium-sized arteries with hardening and loss of elasticity of the arterial walls and narrowing of the arterial lumen. Atherosclerosis is the principal cause of cardiovascular and cerebrovascular diseases leading to angina pectoris, myocardial infarction, and ischemic stroke, and is the principal cause of death in Western countries (>40% of all deaths) (1). The atherosclerotic lesion develops in several stages (2). Initially, fatty streaks are formed, which are characterized by lipid-laden foam cells. Most foam cells arise from monocyte-derived macrophages in the arterial intima, the innermost layer of the arterial wall facing the bloodstream. Fatty streaks may develop into fibrous plaques by smooth muscle cell migration to the intima and proliferation, as well as foam cell necrosis with deposition of extracellular cell debris, lipids, and cholesterol crystals. The advanced stages of atherosclerosis are characterized by mature, fibrous plaques formed by continued proliferation of myointimal cells, necrosis of foam cells and endothelial cells, hemorrhage, mural thrombosis, and calcium deposition (2).

The initiation of atherosclerotic lesion formation is caused by sublethal changes in endothelial function, called endothelial activation or dysfunction (2–4). The vascular endothelium overlying lesion-prone arterial sites shows increased permeability to plasma proteins, including albumin, low-density lipoprotein (LDL), and fibrinogen (2). LDL accumulates in the arterial wall by binding to extracellular matrix proteins of the subendothelial space, including collagen and glycosaminoglycans. In addition, monocytes are preferentially recruited to these lesion-prone areas by attachment to the endothelium and guided migration into the subendothelial space (3). Once in the intima, the monocytes undergo activation and differentiation to resident macrophages under the influence of macrophage colony-stimulating factor released from vascular cells (5, 6). Finally, macrophages are gradually converted to lipid-laden foam cells, the hallmark of the fatty streak.

The initial monocyte-endothelial interactions on arterial prelesion sites are triggered by localized activation of the endothelium and expression of specific cellular adhesion molecules that interact with specific counter receptors on

[1] To whom requests for reprints should be addressed at the Linus Pauling Institute, Oregon State University, 571 Weniger Hall, Corvallis, OR 97331–6512. E-mail: balz.frei@orst.edu

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monocytes (7, 8). Two adhesion molecules that appear particularly important in atherosclerotic lesion development are vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1). VCAM-1 has been shown to be expressed very early, before intimal monocyte-macrophage accumulation, by the endothelium overlying prelesion sites (8), although some studies have reported monocyte accumulation in areas not expressing increased levels of VCAM-1 (9, 10). ICAM-1 is expressed above both lesion and nonlesion areas, and has also been found within lesions (7). In addition to VCAM-1 and ICAM-1, the connecting segment-1 domain of fibronectin (11) and the GRO family of chemokines (12) may also be important in mediating monocyte adhesion to the endothelium in early atherogenesis.

Together with endothelial activation, and closely intertwined with it, oxidative modification of LDL plays a pivotal role in atherogenesis (13–16). The oxidative modification hypothesis of atherosclerosis (13) originated with the observation by Goldstein *et al.* (17) that cultured macrophages are converted to lipid-laden cells in the presence of chemically modified LDL, but not native LDL. Subsequently, Henriksen *et al.* (18) observed that oxidatively modified LDL (ox-LDL) is rapidly internalized by macrophages. Further work revealed that all major cell types present in the arterial wall (i.e., endothelial cells, smooth muscle cells, and macrophages) oxidatively modify LDL (15, 16). The mechanism of oxidative modification of LDL *in vitro* usually involves lipid peroxidation and covalent modification of apolipoprotein B (apo B) by lipid hydroperoxide breakdown products, such as malondialdehyde and 4-hydroxynonenal (14). More recently, evidence has accumulated that myeloperoxidase is present in atherosclerotic lesions and contributes to LDL modification through production of hypochlorous acid (HOCl), tyrosyl radicals, or nitrogen dioxide radicals (15, 19–21). HOCl-mediated LDL modification primarily targets apo B, leaving lipids and associated antioxidants, including α-tocopherol, largely intact (21). HOCl-modified LDL, like ox-LDL, is taken up by macrophages at increased rates, leading to foam cell formation.

Modified LDLs are atherogenic by many additional mechanisms, such as direct chemotactic activity for monocytes, smooth muscle cells, and T-lymphocytes, inhibition of the production and biologic activity of endothelium-derived nitric oxide (EDNO), and cytotoxicity (2, 13–16). Furthermore, minimally oxidized LDL (called minimally modified LDL, mm-LDL) stimulates expression of monocyte chemotactic protein-1 (MCP-1) and granulocyte and macrophage colony-stimulating factors by endothelial cells (5, 22). The critical role of MCP-1 in the initiation of atherosclerosis has been demonstrated recently in MCP-1 null mice lacking the LDL receptor or overexpressing apo B, in which atherosclerotic lesion formation is dramatically reduced compared to mice expressing normal levels of MCP-1 (23, 24). Finally, mm-LDL and ox-LDL stimulate monocyte-endothelial interactions (25–28) and expression of adhesion molecules (11, 12, 28–31). Thus, there appears to be a causal relationship between accumulation and modification of LDL and monocyte recruitment to the arterial wall in the initial stages of atherosclerotic lesion development.

An important implication of the oxidative modification hypothesis of atherosclerosis is that antioxidants may inhibit atherogenesis. We have proposed that antioxidants exert their protective effects against cardiovascular diseases by two fundamental mechanisms (Fig. 1) (32). The first mechanism is LDL-specific antioxidant action i.e., the protection of LDL against oxidative modification by antioxidants present either in LDL, e.g., α-tocopherol and ubiquinol-10, or

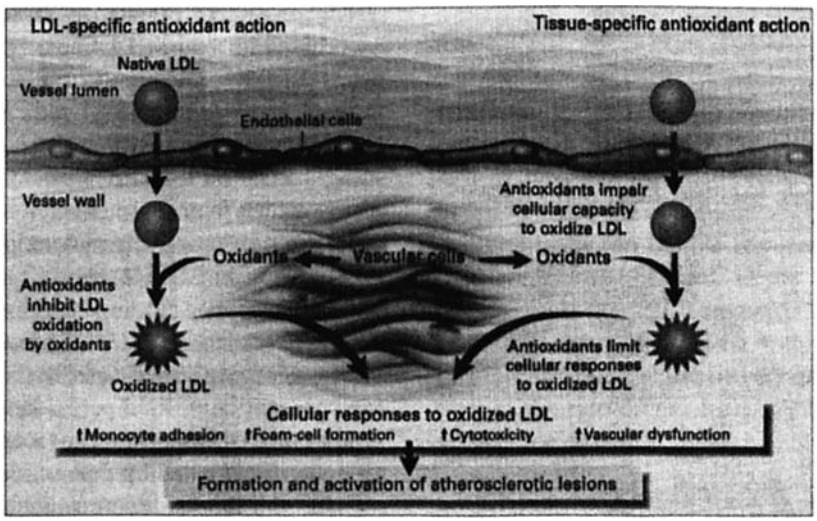

Figure 1. LDL-specific and cell- or tissue-specific mechanisms of antioxidant action. Antioxidants present in LDL or the extracellular fluid of the vascular wall inhibit LDL oxidation. In addition, incorporation of antioxidants into vascular cells inhibits atherosclerosis and reduces the clinical expression of vascular disease by reducing cell-mediated LDL oxidation and the cellular responses to oxidized LDL. This results in decreased monocyte adhesion, foam cell formation, and cell oncosis, and improved vascular function. Small vertical arrows indicate increases. (From Ref. 32 with permission.)

in the extracellular fluid of the subendothelial space, e.g., ascorbic acid (vitamin C) and uric acid. The second mechanism of antioxidant action is tissue- or cell-specific, i.e., increased uptake of antioxidants by vascular cells and increased cellular antioxidant status. This increase in cellular antioxidants may result in decreased production or release of reactive oxygen species and thus less cell-mediated LDL oxidation. In addition, the cellular antioxidants may increase the resistance of vascular cells to the damaging effects of modified LDL. Both the LDL-specific and cell-specific antioxidant actions may lead to decreased adhesion molecule and MCP-1 expression, decreased foam cell formation, and increased EDNO activity, and thus improved vascular function and decreased atherogenesis (Fig. 1).

LDL-Specific Antioxidant Action

Human plasma, the transport medium of LDL, contains a vast array of antioxidant defenses (Table I) (33). There are a number of antioxidant proteins, mainly metal-binding proteins and enzymes, such as extracellular superoxide dismutase, catalase, and glutathione peroxidase. However, these antioxidant enzymes are present in small amounts only in plasma and other extracellular fluids; therefore, the small molecule antioxidants in these fluids are of major importance. The small molecule antioxidants can be separated into water-soluble antioxidants and lipid-soluble or lipoprotein-associated antioxidants. The water-soluble antioxidants include uric acid, ascorbic acid, and bilirubin; glutathione is only present in low concentrations in human plasma, usually 2 μM or less (34), quite in contrast to the intracellular milieu, where glutathione is present in millimolar (1–5 mM) concentrations. The most abundant lipid-soluble antioxidant in LDL is α-tocopherol (14, 35), which is the chemically

Table I. Antioxidant Defenses in Human Plasma and LDL

Antioxidant proteins		
Non-Enzymatic		
Fe- and Cu-binding proteins (albumin, transferrin, ceruloplasmin, etc.)		
Enzymatic		
Superoxide dismutases, catalase, GSH peroxidases: mainly intracellular		
Small molecule antioxidants	Typical plasma concentrations	
Water-soluble	μM	
Uric acid	300	
Ascorbic acid (Vitamin C)	50	
Albumin-bound bilirubin	15	
Glutathione (GSH)	<2	
		mol/mol LDL
Lipid-soluble (lipoprotein-asociated)		
α,γ-Tocopherol (Vitamin E)	25	10
Ubiquinol-10 (coenzyme Q_{10})	1.0	0.4
β-Carotene (Pro-Vitamin A)	0.5	0.2
Lycopene	0.5	0.2

Note. Adapted from Ref. 33.

and biologically most active form of vitamin E. Other LDL-associated antioxidants, such as ubiquinol-10, β-carotene, lycopene, and other carotenoids and oxycarotenoids, are present in much smaller amounts, usually less than $1/20^{th}$ of the α-tocopherol concentration, or less than one molecule per LDL particle (Table I) (14, 35, 36).

Many basic research studies have investigated how effectively and by what mechanisms the endogenous antioxidants in human plasma and LDL inhibit lipid peroxidation and oxidative modification of LDL (14, 37, 38). These studies have focused mainly on three antioxidants, ascorbic acid, α-tocopherol, and β-carotene. We found that among the endogenous plasma antioxidants, ascorbic acid is particularly important in inhibiting lipid peroxidation induced by many different types of oxidative stress, including stimulated neutrophils (39), cigarette smoke (40), enzymatically generated superoxide radicals and hydrogen peroxide (41), aqueous peroxyl radicals (36, 39, 42), and redox active iron (43). Under all of these oxidizing conditions, ascorbic acid is the only antioxidant capable of completely preventing detectable lipid peroxidation in plasma; once ascorbic acid has been depleted, detectable amounts of various classes of lipid hydroperoxides are formed, despite the presence of other plasma antioxidants, including α-tocopherol and β-carotene (36, 39).

We also investigated the protection of isolated human LDL by ascorbic acid against metal ion-dependent and -independent oxidative modification and its underlying mechanisms (44–49). We found that physiologic concentrations of ascorbic acid strongly inhibit LDL oxidation induced by aqueous peroxyl radicals, Cu^{2+}, heme-iron, human aortic endothelial cells, and stimulated neutrophils (44–49). Furthermore, we observed that *in vitro* ascorbic acid is much more effective against Cu^{2+}-induced LDL oxidation than α-tocopherol or β-carotene, both at low and high oxygen partial pressures (46). Other investigators also reported strong protective effects of physiologic concentrations of ascorbic acid against LDL oxidation induced by cultured arterial wall cells, neutrophils, and myeloperoxidase-derived HOCl and tyrosyl radicals (50). Taken together, these studies show that ascorbic acid prevents oxidative modification of LDL *in vitro* by several distinct mechanisms, which may act in concert: 1) free radical scavenging (44, 46), preventing aqueous oxidants from initiating lipid peroxidation in LDL; 2) modification of histidine residues and other copper-binding sites on apo B (47), imparting increased resistance to Cu^{2+}-induced LDL oxidation; 3) destruction of preformed lipid hydroperoxides (45), preventing propagation of lipid peroxidation in LDL; and 4) regeneration of LDL-associated α-tocopherol, thereby inhibiting tocopherol-mediated lipid peroxidation in LDL (see below).

In addition to ascorbic acid, numerous studies have focused on the prevention of LDL oxidation by LDL-associated antioxidants, particularly α-tocopherol (38). *In vitro* supplementation of LDL with α-tocopherol has been shown to inhibit LDL oxidation by endothelial cells, mono-

cyte-macrophages, and Cu^{2+}, whereas *in vitro* supplementation of LDL with β-carotene has yielded inconsistent results (37, 38). A number of clinical studies have investigated the effects of supplementation with lipid-soluble antioxidants *in vivo* on the resistance of plasma-derived LDL to oxidation *in vitro*. Almost all of the studies using α-tocopherol found significantly increased resistance of LDL to oxidation, whereas several investigators, including our laboratory (51), have found no effect of *in vivo* β-carotene supplementation (38). The protective effect of ascorbic acid against LDL oxidation cannot be tested by supplementation *in vivo* followed by isolation of LDL and oxidation *in vitro*, because ascorbic acid is a water-soluble compound, and thus is removed from LDL during isolation from plasma.

Several recent clinical studies have investigated the dose-dependency of the effect of α-tocopherol supplementation on LDL resistance to oxidation. In a placebo-controlled, randomized trial Jialal *et al.* (52) investigated the effects of supplementation for 8 weeks with 60, 200, 400, 800, or 1200 IU/day of α-tocopherol. The minimum dose of vitamin E necessary to significantly decrease the susceptibility of LDL to oxidation was 400 IU/day. Both plasma and LDL α-tocopherol concentrations correlated significantly with inhibition of LDL oxidation. In a similar placebo-controlled trial, Simons *et al.* (53) found that supplementation for 6 weeks with 500, 1000, or 1500 IU vitamin E daily significantly decreased LDL oxidation. One additional study examined the effect of vitamin E dose on the susceptibility of LDL to Cu^{2+}-induced oxidation (54). The design of this study differed from the above two in that each of the subjects ingested consecutively, during 2-week periods, increasing amounts of vitamin E of 25, 50, 100, 200, 400, and 800 IU/day. LDL vitamin E concentrations increased linearly with dose as did the resistance of LDL to oxidation. Even at the lowest dose of vitamin E, 25 IU/day, a significant increase in the resistance of LDL to oxidation was observed. These results contrast with those of Jialal *et al.* (52) in which a dose of 400 IU/day was required for a significant decrease of *in vitro* LDL oxidation.

The discrepancies between these two studies (52, 54) and several others in the literature may be explained by the fact that LDL-associated α-tocopherol can act as a pro-oxidant *in vitro*, initiating and propagating lipid peroxidation, rather than inhibiting it (55). Stocker and co-workers have shown that at low radical flux rates and in the absence of co-antioxidants such as ubiquinol-10 and ascorbic acid, LDL-associated α-tocopherol behaves as a pro-oxidant. Under these conditions, α-tocopherol acts both as a phase-transfer and chain-transfer reagent (Fig. 2). Phase-transfer of radicals occurs when α-tocopherol in LDL reacts with radical oxidants in the aqueous phase, resulting in the formation of an α-tocopheroxyl radical. The α-tocopheroxyl radical, due to lack of another radical in LDL to react with, eventually extracts a hydrogen atom from a polyunsaturated fatty acid, leading to the formation of a carbon-centered

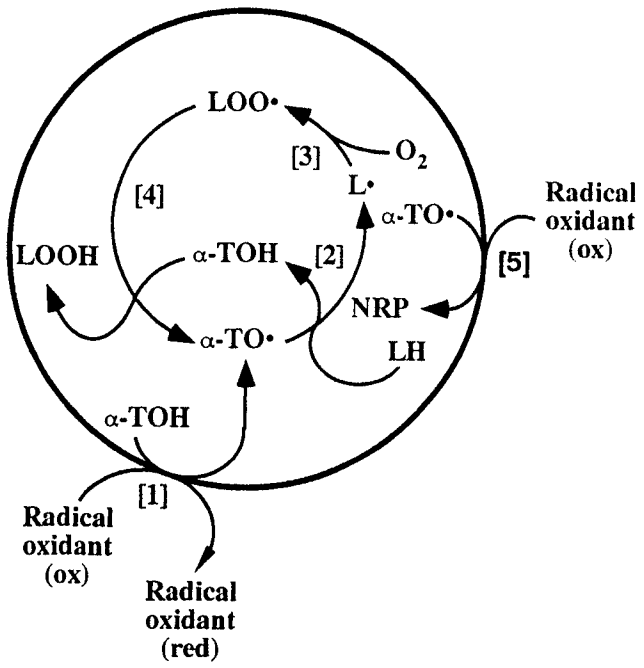

Figure 2. Tocopherol-mediated peroxidation (TMP) of LDL *in vitro*. A solution of radical oxidizing LDL is an aqueous emulsion of lipid particles where the radical in one oxidizing particle, present predominantly as an α-tocopheroxyl radical (α-TO•), is segregated from α-TO• in other oxidizing particles, and oxidation of the lipids proceeds *via* TMP. TMP is initiated by reaction [1], reflecting the phase-transfer activity of α-tocopherol (α-TOH). Lipid peroxidation initiation (reaction [2]) followed by the propagation reactions [3] and [4] reflect the chain-transfer activity of α-TOH. Inhibition of TMP is achieved by reaction of a second aqueous radical oxidant with α-TO• (reaction [5]), resulting in both formation of nonradical product(s) (NRP) and consumption of α-TOH. Reaction [5] predominates over the chain-transfer activity under conditions of high radical flux, characterized by rapid consumption of α-TOH, whereas reactions [2]–[4] predominate under mild oxidizing conditions (i.e., when α-TOH is consumed slowly). It is assumed that lipid peroxyl radicals (LOO•) and α-TO• move freely within lipoproteins, whereas they do not readily escape from the particles due to their hydrophobicity. LH, lipid molecule containing a polyunsaturated fatty acyl side chain; L•, carbon-centered lipid radical; LOOH, lipid hydroperoxide. (From Neuzil J, Thomas SR, Stocker R. Requirement for, promotion, or inhibition by α-tocopherol of radical-induced initiation of plasma lipoprotein lipid peroxidation. Free Radic Biol Med **22**:57–71, 1997, with permission.)

radical and regenerating α-tocopherol (Fig. 2). The carbon-centered radical then undergoes oxidation to a lipid peroxyl radical, which is scavenged by α-tocopherol to form a lipid hydroperoxide molecule and another α-tocopheroxyl radical. The latter can initiate another round of lipid peroxidation, completing the cycle of the chain-transfer reaction of α-tocopherol (Fig. 2). The results of *in vitro* LDL oxidation studies therefore depend on both the α-tocopherol content of LDL and the specific incubation conditions used, in particular, the rate of radical production and the presence or absence of co-antioxidants.

Cell-Specific Antioxidant Action

In addition to protecting LDL directly against oxidation, ascorbic acid, α-tocopherol, and other antioxidants may inhibit cell-mediated LDL oxidation by reducing the cellular production and release of reactive oxygen species,

including superoxide radicals and hydrogen peroxide. Supplementation of cultured endothelial cells and smooth muscle cells with α-tocopherol or β-carotene results in decreased minimal modification of LDL (22). Furthermore, Parthasarathy (56) reported that loading of endothelial cells or macrophages with a water-soluble derivative of probucol, a potent antioxidant, markedly decreased LDL oxidation by these cells. Two recent studies reported that enrichment of monocytes with α-tocopherol caused decreased production of superoxide radicals (57), and peripheral monocytes isolated from human subjects supplemented with α-tocopherol exhibited decreased superoxide radical production and interleukin (IL)-1β release (58). Finally, we have recently shown that loading of human aortic endothelial cells with ascorbic acid reduces production of reactive oxygen species and inhibits cell-mediated oxidative modification of LDL (49).

In addition to modulating the cellular capacity to oxidize LDL, cellular antioxidants may affect the response of vascular cells to mm-LDL or ox-LDL, as manifested by either activation (e.g., increased endothelial MCP-1 and adhesion molecule expression and decreased EDNO production) or apoptosis and oncosis. As discussed above, endothelial activation leading to monocyte recruitment to the arterial wall is one of the earliest events in atherogenesis, whereas apoptosis or oncosis of foam cells, smooth muscle cells, and endothelial cells play important roles in lesion progression.

Inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and IL-1β are well known to upregulate cellular adhesion molecules in endothelial cells, such as VCAM-1 and ICAM-1 (4, 7, 8, 59). It has been suggested that these inflammatory cytokines as well as modified LDL activate endothelial cells by a mechanism involving reactive oxygen species. TNF-α and other inflammatory cytokines may stimulate intracellular production of superoxide radicals and hydrogen peroxide, which may act as second messengers to activate the transcription factor nuclear factor κB (NFκB) (4, 60, 61). Activation and nuclear transfer of NFκB are known to induce adhesion molecule and MCP-1 expression in endothelial cells (60, 61).

The likely involvement of modified LDL and intracellular reactive oxygen species in upregulation of cell adhesion molecules led to the hypothesis that antioxidants may inhibit this process. Cominacini *et al.* (30) reported that pretreatment of human umbilical vein endothelial cells with vitamin E or probucol significantly reduced ox-LDL-induced VCAM-1 expression. All of the other studies investigating the effects of antioxidants on upregulation of adhesion molecules and monocyte adhesion have used inflammatory cytokines, not modified forms of LDL. Faruqi *et al.* (62) demonstrated an inhibitory effect of cellular α-tocopherol on IL-1β-induced upregulation of E-selectin and monocyte adhesion to endothelial cells. Recently, α-tocopherol enrichment of monocytes was shown to decrease agonist-induced adhesion to endothelial cells (57). Probucol and *N*-acetylcysteine also inhibited monocyte adhesion induced by IL-1β, whereas extracellular vitamin C had no effect (62, 63). Marui *et al.* (59) found that the thiol antioxidants *N*-acetylcysteine and pyrrolidine dithiocarbamate inhibit cytokine-induced VCAM-1 expression in endothelial cells, but ICAM-1 and E-selectin expression were either not affected or only little affected by pyrrolidine dithiocarbamate. In agreement with these findings, Weber *et al.* (61) reported that *N*-acetylcysteine and pyrrolidine dithiocarbamate suppress VCAM-1 expression in human umbilical vein endothelial cells exposed to TNF-α. These authors also demonstrated that endothelial cells produce superoxide radicals in response to TNF-α, possibly as an intracellular messenger molecule, and thus suggested that antioxidants may act by disrupting intracellular signaling pathways.

Extracellular ascorbic acid also has been reported to inhibit neutrophil-endothelial cell interactions (64, 65). We have shown that physiologic concentrations of ascorbic acid abolish cigarette smoke- or ox-LDL-induced leukocyte adhesion to endothelium *in vivo* (66, 67). In addition, smokers have lower plasma levels of vitamin C, and monocytes isolated from smokers exhibit increased adhesiveness to cultured human umbilical vein endothelial cells (68). Following supplementation of the smokers with vitamin C, their monocytes exhibited normal endothelial adhesiveness. Thus, like vitamin E (57), vitamin C may not only affect adhesion molecule expression on endothelial cells (69), but also activation of counter receptors on monocytes, such as the integrin CD11b/CD18 (68). However, in another study, supplementation of smokers with vitamin C was without effect on *ex vivo* monocyte–endothelial interactions (70). Interestingly, supplementation with L-arginine, the physiologic substrate for nitric oxide synthase, significantly reduced monocyte-endothelial cell adhesion, suggesting an important role for EDNO (see also below).

Although moderate oxidative stress may cause cellular dysfunction by activation of redox-sensitive transcription factors, more severe oxidative stress may result in apoptosis or oncosis. Cells with an increased antioxidant status should be more resistant to a cytotoxic oxidant challenge. It has been demonstrated that increasing cellular α-tocopherol or probucol content inhibits the cytotoxic effects of ox-LDL (71, 72). In addition, it is well established that intracellular glutathione plays a central role in protecting endothelial cells against ox-LDL and other oxidant insults, such as hydrogen peroxide (73–76). Kaneko *et al.* (77) failed to observe a protective effect of pre- or co-incubation of endothelial cells with ascorbic acid against lipid hydroperoxide–induced cytotoxicity.

In addition to affecting cell-mediated LDL oxidation, adhesion molecule and MCP-1 expression, and oncosis, cellular antioxidants also play an important role in maintaining the normal production and biologic activity of EDNO. Nitric oxide is of central importance in both atherosclerosis and its clinical manifestations (i.e., angina pectoris, myocardial infarction, and stroke) as it inhibits endothelial-

leukocyte interactions, smooth muscle cell proliferation, and platelet aggregation, and causes vasodilation (78). It is known that under conditions of oxidative stress, NO is inactivated by two basic mechanisms. First, superoxide radicals react rapidly with NO, which limits the biological activity of EDNO (79). Superoxide radicals are produced at increased rates in atherosclerosis and hypercholesterolemia (80). The second mechanism involves ox-LDL, which is present in atherosclerotic lesions and can react directly with and thereby inactivate NO (81). In addition, ox-LDL can interrupt the agonist-dependent NO production by endothelial cells (82).

Since oxidative stress inhibits the biological activity of NO, antioxidants may be protective. Numerous recent studies have reported beneficial effects of vitamin C, administered either orally or by intra-arterial infusion, on vasodilation in various patient groups (Table II). We and others (83–85) have found that vasodilation in patients with cardiovascular disease is significantly improved following treatment with vitamin C, and is comparable to vasodilation seen in healthy control subjects. Similar beneficial effects of vitamin C with normalization of the vasodilatory response were observed in patients with coronary spastic angina (86), chronic heart failure (87), hypercholesterolemia (88), or hypertension (89, 90), as well as in healthy volunteers following methionine loading to cause hyperhomocysteinemia (91) (Table II). Heitzer *et al.* (92) and Motoyama *et al.* (93) observed significantly improved vasodilation in smokers given vitamin C infusions. In addition, patients with non-insulin-dependent and insulin-dependent diabetes mellitus demonstrated increased blood flow after infusion of vitamin C (94, 95). Finally, healthy individuals given an oral dose of 1000 mg of vitamin C in combination with 800 IU of vitamin E exhibited normal vasodilation several hours after a single high fat meal, whereas control subjects not given the antioxidant combination showed impaired vasoreactivity (96). Vitamin E alone also has been shown to improve endothelium-dependent vasodilation in various patients (97–99), although a recent study found that vitamin E supplementation does not improve vasodilation in older adults (100).

Several mechanisms may underlie the salubrious ef-

Table II. Studies Showing Significant Improvement of Endothelium-Dependent Vasodilation Following Vitamin C Treatment in Humans

Reference and parameter measured	Subjects	Vitamin C dose
94: forearm blood flow	10 patients: type 2 diabetes 10 control subjects	24 mg/min (infusion)
83: brachial artery dilation	46 coronary artery disease patients 20 control subjects	2000 mg (oral, 2 hr); plasma concentration 2.5-fold increased
92: forearm blood flow	10 chronic smokers 10 control subjects	18 mg/min (infusion)
93: brachial artery dilation	20 smokers 20 nonsmokers	10 mg/min (infusion)
88: forearm blood flow	11 hypercholesterolemic patients 12 control subjects	24 mg/min (infusion)
89: coronary artery dilation	22 hypertensive patients 5 control subjects	3000 mg (infusion)
87: radial artery dilation	15 chronic heart failure patients 8 control subjects	25 mg/min (infusion), 2000 mg/day (oral, 4 wk)
95: forearm blood flow	10 patients: type 1 diabetes 10 control subjects	24 mg/min (infusion)
86: epicardial artery dilation	32 coronary spastic angina patients 34 control subjects	10 mg/min (infusion)
90: forearm blood flow	14 essential hypertensive patients 14 control subjects	24 mg/l forearm tissue/min (infusion)
85: brachial artery dilation	10 coronary artery disease patients 10 control subjects	1000 mg (infusion)
84: brachial artery dilation	46 coronary artery disease patients 25 control subjects	2000 mg (oral, 2 hr); plasma concentration 2.8-fold increased 500 mg/day (oral, 4 wk); plasma concentration 2.3-fold increased
91: brachial artery dilation	17 healthy volunteers before and after methionine load (hyperhomocysteinemia) or placebo	1000 mg/day (oral, 1 wk)

Note. Adapted from Carr AC, Frei B. Toward a new recommended dietary allowance for vitamin C based on antioxidant and health effects in humans. Am J Clin Nutr **69**:1086–1107, 1999.

fects of vitamin C on vasodilation. Ascorbic acid may spare NO by scavenging superoxide radicals or preventing the formation of ox-LDL. However, the latter mechanism is unlikely to be relevant because the effects of vitamin C administration on vasodilation are observed within hours (Table II), a period too short to affect *in vivo* LDL oxidation. With respect to the former mechanism, it should be noted that high concentrations of vitamin C are required to scavenge superoxide radicals in competition with NO (101) due to the large difference in rate constants (rate constant for the reaction between superoxide radicals and ascorbate, 2×10^5 $M^{-1}s^{-1}$ at pH 7.4; rate constant for the reaction between superoxide radicals and NO, 1.7×10^{10} $M^{-1}s^{-1}$). Nevertheless, millimolar plasma concentrations of vitamin C are achievable with infusion, and intracellular ascorbic acid concentrations are in the millimolar range (102). Therefore, superoxide radical scavenging may, at least in part, explain the beneficial effects of vitamin C on vasodilation. Vitamin C can also maintain high intracellular levels of glutathione, primarily by a sparing effect (103), which may enhance the synthesis of EDNO or increase the stabilization of NO *via* formation of S-nitrosothiol species (83).

In support of the notion that cellular glutathione levels play an important role in EDNO biologic activity, we have observed that administration of L-2-oxothiazolidine-4-carboxylic acid, a cellular cysteine delivery agent known to increase intracellular glutathione levels, enhances vasodilation in patients with coronary artery disease (104). Glutathione, which like ascorbic acid is present intracellularly in millimolar concentrations, may also inhibit inactivation of EDNO by scavenging superoxide radicals. In addition, glutathione may directly stimulate endothelial nitric oxide synthase, which has been shown to be the case for the neuronal enzyme (105). Finally, either ascorbic acid or glutathione may inhibit oxidation of tetrahydrobiopterin, which is a cofactor for the nitric oxide synthase reaction, or stimulate endothelial L-arginine uptake, which is the substrate for nitric oxide synthase. Most interestingly, a recent study demonstrated that intracellular ascorbic acid potentiates NO synthesis by endothelial cells, and that both L-arginine uptake and nitric oxide synthase expression were unaffected (106). The authors concluded that ascorbic acid likely enhances the availability of tetrahydrobiopterin or increases its affinity for the endothelial nitric oxide synthase (106).

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