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FORUM REVIEW ARTICLE

Role of Vitamin C in the Function of the Vascular Endothelium

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Abstract

Significance: Vitamin C, or ascorbic acid, has long been known to participate in several important functions in the vascular bed in support of endothelial cells. These functions include increasing the synthesis and deposition of type IV collagen in the basement membrane, stimulating endothelial proliferation, inhibiting apoptosis, scavenging radical species, and sparing endothelial cell-derived nitric oxide to help modulate blood flow. Although ascorbate may not be able to reverse inflammatory vascular diseases such as atherosclerosis, it may well play a role in preventing the endothelial dysfunction that is the earliest sign of many such diseases. ***Recent Advances:*** Beyond simply preventing scurvy, evidence is mounting that ascorbate is required for optimal function of many dioxygenase enzymes in addition to those involved in collagen synthesis. Several of these enzymes regulate the transcription of proteins involved in endothelial function, proliferation, and survival, including hypoxia-inducible factor-1α and histone and DNA demethylases. More recently, ascorbate has been found to acutely tighten the endothelial permeability barrier and, thus, may modulate access of ascorbate and other molecules into tissues and organs. ***Critical Issues:*** The issue of the optimal cellular content of ascorbate remains unresolved, but it appears that low millimolar ascorbate concentrations are normal in most animal tissues, in human leukocytes, and probably in the endothelium. Although there may be little benefit of increasing near maximal cellular ascorbate concentrations in normal people, many diseases and conditions have either systemic or localized cellular ascorbate deficiency as a cause for endothelial dysfunction, including early atherosclerosis, sepsis, smoking, and diabetes. ***Future Directions:*** A key focus for future studies of ascorbate and the vascular endothelium will likely be to determine the mechanisms and clinical relevance of ascorbate effects on endothelial function, permeability, and survival in diseases that cause endothelial dysfunction. *Antioxid. Redox Signal.* 19, 2068–2083.

Introduction

Vitamin C, or ascorbic acid, is required to prevent scurvy, but debate continues as to whether any single function of the vitamin is really necessary and the extent to which ascorbate contributes to optimal function of an organ or even a cell. One of the organs most affected by ascorbate is the endothelium, which regulates the distribution of ascorbate throughout the body and where ascorbate has many functions. Ascorbate has long been known to enhance endothelial synthesis and deposition of Type IV collagen to form the basement membrane of blood vessels. More recent studies reveal other potential functions of the vitamin in the endothelium, especially as related to control of endothelial cell proliferation and apoptosis, smooth muscle-mediated vasodilation, and endothelial permeability barrier function. Accordingly, this review will consider the extent to which ascorbate helps maintain the health of the endothelium, the mechanisms by which it does so, and how ascorbate might aid in the normal functions of the endothelium.

Ascorbate Chemistry and Biochemical Functions

Ascorbate chemistry

As shown in Figure 1, four of the six ascorbic acid carbons form a cyclic 5-membered lactone ring that is strained because

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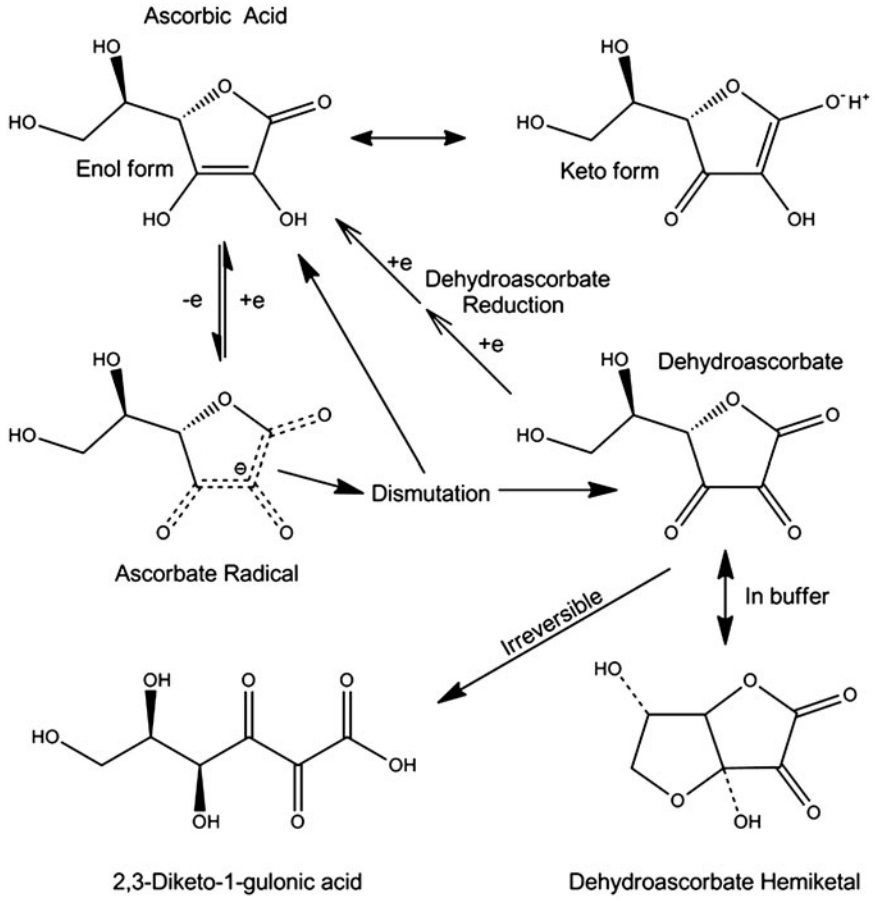

FIG. 1. Ascorbic acid metabolism. Ascorbate donates a single electron to become the ascorbate radical, which reacts with another ascorbate radical to form a molecule each of ascorbate and dehydroascorbate (DHA). The latter is unstable at physiologic pH and if not reduced back to ascorbate *via* GSH-dependent mechanisms, it will undergo irreversible ring opening and loss. In buffers, DHA forms a hemiketal that has a molecular structure resembling that of glucose.

of carbon bond angle preferences. Although aliphatic alcohols are usually not acidic, the presence of a double bond between carbons 2 and 3 allows for keto-enol tautomerism, lowering the pK_a of ascorbic acid to 4.1 (Fig. 1). Thus, it is effectively a monoanion at physiologic pH. Ascorbate donates a single electron in all its redox reactions, generating the ascorbate radical. This radical is not very reactive with anything but itself (17). Dismutation of two ascorbate radicals forms a molecule each of ascorbate and dehydroascorbate (Fig. 1). Dehydroascorbate, a tri-ketone lactone ring structure, is very unstable, with a half life in physiologic buffer of about 6 min (47, 178). Hydrolysis of the lactone ring irreversibly converts it to 2,3-diketo-1-gulonic acid (Fig. 1) (19, 30). In buffer, dehydroascorbate preferentially forms a hemiketal (43, 126) (Fig. 1) that resembles glucose in its molecular configuration and has affinity for the GLUT-type glucose transporter (165).

Ascorbate uptake

Since humans cannot synthesize their own vitamin C, it should be absorbed in the intestine and carried through the circulation to the various organs (Fig. 2). The vitamin is taken up as ascorbate into intestinal cells on a dedicated sodium- and energy-dependent transporter, termed the Sodium-dependent Vitamin C Transporter 1 (SVCT1). Dehydroascorbate uptake on the intestinal Sodium-dependent Glucose Transporter-1 (SGLT1) may also contribute to absorbed ascorbate (16). Ascorbate probably exits the enterocytes *via* an unknown transporter (Fig. 2, left side) and somehow enters the circulation where it typically circulates at concentrations of 40–60 μM, depending on dietary intake and rates of disposition. Due to its low molecular weight, vitamin C is freely filtered by the kidney, but reabsorbed in the renal proximal tubule, again by the SVCT1 (Fig. 2, left side). This mechanism helps conserve ascorbate in the blood for uptake into the tissues. Cellular uptake of ascorbate occurs largely on the Sodium-dependent Vitamin C Transporter 2 (SVCT2), which is closely related to the SVCT1 in structure and function (161), but is located mostly in non-epithelial cells (Fig. 2). It is the only known ascorbate transporter in endothelial cells (141). This transporter generates a sharp gradient of ascorbate from the blood to human white blood cells, resulting in intracellular concentrations of the vitamin as high as 3 mM in monocytes (14) and 2 mM in neutrophils (173) and platelets (53). Mature erythrocytes, which do not contain either SVCT (109), have the same intracellular ascorbate concentrations as in the surrounding blood plasma (Fig. 2, center). Ascorbate exits from the bloodstream, as discussed in greater detail next, then enters various cells and organs, again largely on the SVCT2 (Fig. 2, right side).

The intracellular ascorbate concentration in endothelial cells has not been directly measured, but based on levels that can be achieved by adding ascorbate to cultured cells, it is likely in the range of 2–4 mM (95, 103). Perhaps, of significance, such low millimolar concentrations are required for optimal synthesis of type IV collagen (103). As expected, the SVCT2 is expressed in primary culture endothelial cells derived from both pigs (15) and mice (131). However, not all

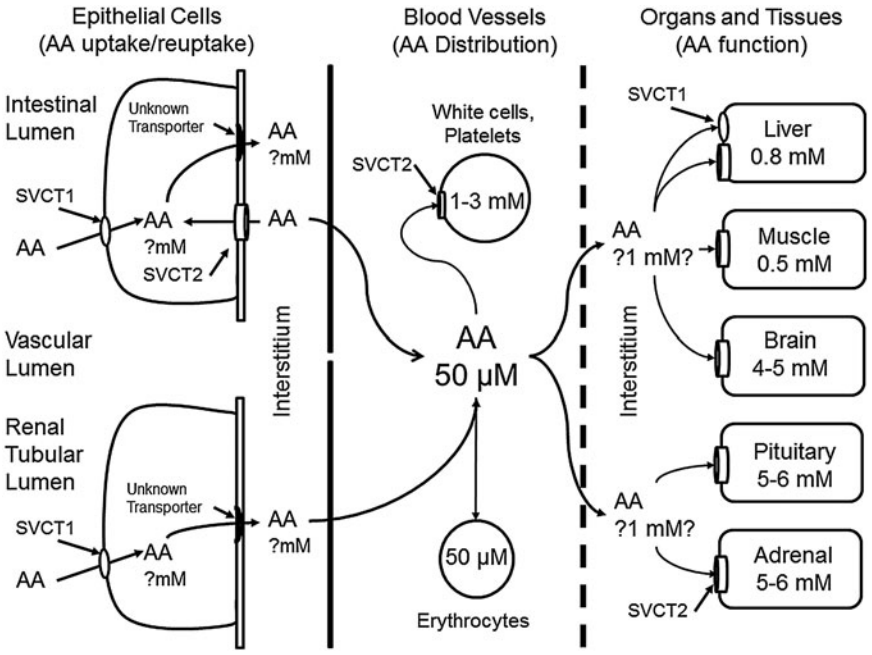

FIG. 2. Uptake and distribution of ascorbate across the vascular bed. Ascorbate (AA) is taken up from the intestine either on the SVCT1 or as DHA on glucose transporters (not shown). Once inside the intestinal epithelium, it exits by an unknown mechanism on the basolateral membrane into the interstitium and then into nearby capillaries. Ascorbate in the bloodstream is taken up by erythrocytes (either as DHA or as slow diffusion) and by leukocytes and endothelial cells on the SVCT2. Plasma ascorbate is distributed by the vascular tree to organ beds. Interstitial ascorbate is then taken up by the SVCT2 on nucleated cells in the organs. In the central nervous system, ascorbate enters the cerebrospinal fluid largely by secretion from the choroid plexus (not shown). SVCT1, sodium-dependent vitamin C transporter 1; SVCT2, sodium-dependent vitamin C transporter 2.

endothelial cells express the SVCT proteins. Endothelial cells forming the blood–brain barrier appear to lack the SVCT2 *in vivo* (57) and at least initially *ex vivo* (131). Perhaps in line with this, ascorbate does not cross the blood–brain barrier *in vivo* at appreciable rates (2). Although lack of the SVCT2 might at first glance be considered the cause of blood–brain barrier impermeability to ascorbate, as discussed next, ascorbate in other vascular beds likely crosses the endothelial barrier by diffusion around the cells, which is severely limited by the tight junctions of the blood–brain barrier.

Ascorbate can also enter cells *via* its two-electron-oxidized form, dehydroascorbate (Fig. 3). As noted earlier, the hemiketal of dehydroascorbate (Fig. 1) is transported into cells by facilitated diffusion on the ubiquitous GLUT-type glucose transporters. Once inside the cell, dehydroascorbate is rapidly reduced back to ascorbate, However, given that plasma dehydroascorbate concentrations are usually 2 μM or less (42), and that the normal circulating level of 5 mM glucose will compete for uptake with dehydroascorbate, it is unlikely that ascorbate concentrations in cells are bolstered much by dehydroascorbate, except perhaps in erythrocytes or in conditions of high oxidative stress in blood, such as during the respiratory burst of phagocytic cells (124, 172). Additional evidence suggesting that the SVCT2 may be the only route of ascorbate entry in most cells is that brains of embryonic mice lacking both copies of the SVCT2 have undetectable ascorbate levels (146), as do primary culture macrophages lacking the SVCT2 (8).

Ascorbate recycling

Once inside cells, ascorbate is essentially trapped due to its hydrophilic nature and negative charge. Intracellular ascorbate can then scavenge a variety of radicals, with superoxide being one of the most important. Superoxide might be generated within the cell as a result of receptor activation, such as by advanced glycation end products or by thrombin (Fig. 3). Superoxide can also originate as a byproduct of mitochondrial metabolism, especially in response to excessive glucose metabolism in diabetes. The major scavenger of superoxide in cells is likely to be superoxide dismutase, which has been noted to react *in vitro* with superoxide 10^5 times faster than ascorbate (78) (see Table 1 for rate constants). In the endothelial cell, there will also be plentiful nitric oxide, which will

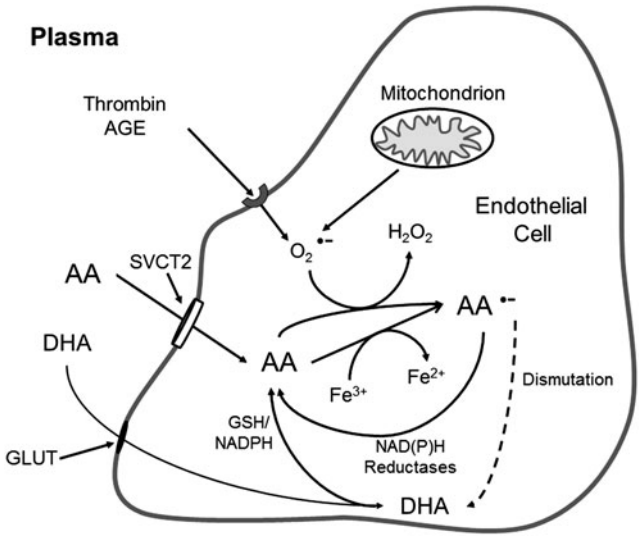

FIG. 3. Endothelial cell ascorbate uptake and recycling. Ascorbate (AA) enters endothelial cells largely on the SVCT2, although a small amount may come in as DHA on glucose transporters (GLUT), to be rapidly reduced to ascorbate in the cell. Once in the cell, ascorbate can donate an electron ferric iron, superoxide ($O_2^{\bullet-}$), and other radical species generated in mitochondria or *via* activation of cell surface receptors, such as those for thrombin or advanced glycation end-products (AGE). The resulting ascorbate radical ($AA^{\bullet-}$) is mostly reduced directly back to ascorbate by NADH- and NADPH-dependent reductases. However, if the oxidative stress is overwhelming, the ascorbate radical may dismutate to form ascorbate and DHA, with subsequent reduction of the latter back to ascorbate.

Table 1. Rate Constants for Reaction of Antioxidants with Superoxide and Peroxynitrite

Antioxidant	*Superoxide* ($M \cdot s^{-1}$)	*Reference*	*Peroxynitrite* ($M \cdot s^{-1}$)	*Reference*
Superoxide dismutase	2×10^9	(56)	—	
Ascorbate	3.3×10^5	(62, 123)	236	(148)
GSH	10^2–10^3	(180)	580	(144)
α-Tocopherol[a]	4900	(62)	6600	(61)
Tetrahydrobiopterin	3.9×10^5	(164)	6000	(83)
Uric acid	[b]		155–700	(84, 147)

[a]Determined in phospholipid micelles and only estimates of bimolecular rate constants.
[b]Uric acid does not appear to react directly with superoxide (12, 84), but the uric acid radical rapidly does so with a rate constant of 8×10^8 $M \cdot s^{-1}$ (137).

react at diffusion-limited rates with superoxide [rate constant $0.7–1.9 \times 10^{10}$ $M \cdot s^{-1}$ (76)], generating the strong oxidant peroxynitrite (13). Nonetheless, ascorbate at concentrations of 1–10 m*M* does scavenges superoxide and enhances arterial relaxation to acetylcholine in rabbit thoracic aorta (78), suggesting that the presumed low millimolar ascorbate concentrations in endothelial cells may allow ascorbate to aid in scavenging both superoxide (78) and peroxynitrite (78, 83). Ascorbate can also reduce enzyme-bound ferric to ferrous iron in enzymes (Fig. 3), as discussed in detail next.

In all these reactions, the resulting ascorbate radical is rapidly reduced back to ascorbate by one or more poorly characterized NADH-dependent dehydrogenases (67, 77, 91), as well as by thioredoxin reductase using NADPH as the reducing agent (98) (Fig. 3). As noted earlier in Figure 1, two ascorbate radicals can also dismutate to form a molecule each of ascorbate and dehydroascorbate. The latter is reduced directly *via* a two-step mechanism by reduced glutathione, or enzymatically by GSH-dependent thiol transferases (174, 179) or by NADPH-dependent reductases (41), the latter again including thioredoxin reductase (100) (Fig. 3).

Ascorbate transfer across the endothelial barrier

The barrier to diffusion of substances out of blood vessels varies with vessel type (artery, vein, or capillary) and tissue bed. Indeed, the tightness of the endothelial permeability barrier changes along the vascular tree, from well-organized and numerous connections between endothelial cells in large arteries and veins (where there is strict control of permeability), to near absence in some postcapillary venules, where exchange of plasma and interstitial constituents is efficient (40). Endothelial cells in some organs, such as those in liver sinusoids and the renal glomerulus, have fenestrations that clearly facilitate such exchange. In larger vessels or vascular beds where the endothelium presents a substantial barrier to the exchange of both large and small molecules, ascorbate could enter the interstitium by transiting either through endothelial cells or going between them.

Ascorbate readily enters most endothelial cells on the luminal SVCT2. However, ascorbate is also known to efflux from cultured endothelial cells (104, 162), an effect enhanced by increases in intracellular calcium (37). It follows that this efflux could reflect vectorial ascorbate efflux from the abluminal or basolateral side of the cells (104). This model would mirror that which exists for epithelial cell barriers (Fig. 2), which are much tighter than those generated by the endothelium. For example, the colon carcinoma-derived cell line Caco-2, which has been used to model the intestinal epithelial barrier, expresses the SVCT1 in the apical cell membrane (20, 96). The SVCT2 isoform is expressed on the basolateral membrane (20). Both transporters are thought to mediate unidirectional ascorbate transport (161); so, their orientation in this manner would serve only to bring ascorbate into the cells. Unless the functional orientation of the SVCT2 can reverse, this would require a yet-to-be-described transporter or channel to facilitate ascorbate efflux from the basolateral cell border.

At least in endothelial cells that form a permeability barrier, it appears that ascorbate crosses this barrier by going around the cells in a paracellular manner (108) (Fig. 4). The evidence for this is that intracellular ascorbate did not appreciably efflux below endothelial cells cultured on semi-porous filters for more than 90 min. Indeed, there was an efflux of intracellular ascorbate into the luminal, not the abluminal compartment. Further, ascorbate accumulation below the cells and filters was decreased rather than increased as the intracellular ascorbate concentration was increased (108). It was concluded that short-term ascorbate transfer occurs on gaps between the cells or even across one or more cellular junctional connections (Fig. 4). Endothelial cells in culture have 0.5–2 μm gaps between as many as 10% of cells, which decrease in size with time in culture (3, 108). Ascorbate could, thus, diffuse across

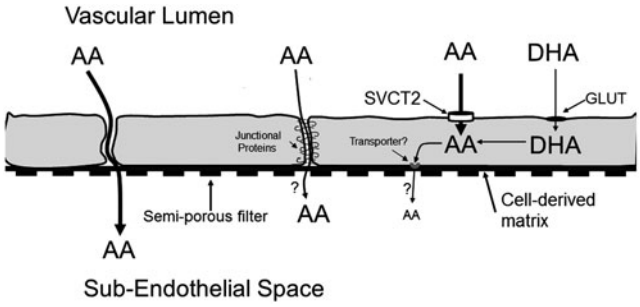

FIG. 4. Routes of transfer of ascorbate out of the vascular bed as represented by endothelial cells in culture on semi-porous filters. Ascorbate (AA) or DHA added on the luminal side of endothelial cells cultured on semi-porous membranes enter the cells on the SVCT2 or GLUT-type transporter, respectively. The resulting ascorbate is trapped with little exit on the basolateral side of the cells over a 90 min time-frame. Rather, most ascorbate passes between the cells by a paracellular route, which intracellular ascorbate tightens. There may also be some transit of ascorbate between the cells as sieving across tight junctional proteins.

any gaps between the cells with little sieving effect. Ascorbate could also diffuse across the cellular connections that form rings of attachment between endothelial cells (4) (Fig. 4). Cell-to-cell endothelial connections are made up of tight junctions, gap junctions, adherence junctions, and syndesmos (11, 40), although they are not as tight as those found in epithelial layers (3). These junctions have been proposed as a barrier that contains pores and transporters which might regulate the passage of small charged molecules such as ions (11, 155). Our previous finding that ascorbate transfer across an endothelial cell barrier was decreased by several anion channel inhibitors, including sulfinpyrazone, 5-nitro-2-(3-phenylpropylamino)-benzoic acid, niflumic acid, and probenecid (108), suggests that such a mechanism might apply to ascorbate.

If ascorbate leaves the vascular space by diffusion between endothelial cells as suggested by experiments using cells cultured on semi-permeable membranes, then the interstitial ascorbate concentration will be no greater than the plasma concentration and will decrease near cells taking ascorbate up on the SVCT2. However, direct measurement of subcutaneous dermal ascorbate concentrations by microdialysis coupled with gas chromatography-mass spectroscopy showed an interstitial ascorbate concentration of 1 m*M* (87). This ascorbate concentration is similar to that generated by the SVCT2 in many cell types and is 15–20-fold higher than present in blood (Fig. 2). If correct, this measurement suggests that the simple diffusion of ascorbate which we observed between dermal microcapillary endothelial cells in primary culture (108) does not reflect the situation *in vivo*. Rather, it suggests that the normal dermal capillary endothelial barrier is very tight to ascorbate passage and that there is an as-yet-undiscovered secretory component from the basolateral endothelium (or from cells in the interstitial space) which is not detected in short-term cell culture experiments.

Ascorbate Function in Endothelial Cells

Antioxidant function: scavenging of endogenous and exogenous radicals

Ascorbate has long been considered a key cellular antioxidant, serving as a primary antioxidant by detoxifying exogenous radical species that have entered cells or which have arisen within cells due to excess superoxide generation by mitochondrial metabolism, by NADPH oxidase, xanthine oxidase, or by uncoupled nitric oxide synthase (NOS). As previously noted, at the low millimolar concentrations of ascorbate likely to exist in endothelial cells, ascorbic acid will aid superoxide dismutase in scavenging superoxide and its more toxic breakdown products (Fig. 3). The question arises as to just how much intracellular ascorbate contributes to the antioxidant capacity of cells. GSH is often thought of as the major low-molecular-weight antioxidant in cells, both scavenging radicals and serving as the electron donor for antioxidant enzymes such as glutathione peroxidase. It is certainly a stronger reducing agent than ascorbate (24) and even recycles ascorbate, as noted earlier. However, GSH is more than 100-fold less reactive with superoxide than is ascorbate (Table 1) and although GSH is converted to GSSG, the overall reaction does not reduce superoxide to H_2O_2 (180). The reaction rate of peroxynitrite with GSH is twice that of peroxynitrite with ascorbate (Table 1), so it should effectively scavenge the oxidant. Thus, although GSH might be a more effective scavenger of peroxynitrite than is ascorbate, only ascorbate will effectively remove superoxide.

It is likely that endothelial cells treated with ascorbate concentrations similar to those found in plasma will have similar intracellular ascorbate and GSH concentrations, both of which are in the low millimolar range (102, 107). Further, ascorbate concentrations decrease at lower levels of oxidative stress than do GSH concentrations when these are generated by several oxidants, including menadione (110), ferricyanide (101), and oxidized low-density lipoprotein (LDL) (99). Although these results might suggest that ascorbate "takes the first hit" in the defense against reactive oxygen or nitrogen species, ascorbate could also appear to be more sensitive to oxidative stress than GSH, because the latter has a more robust recycling mechanism and as GSH can be synthesized by the cell.

The reaction rates of superoxide and peroxynitrite with three other cellular antioxidants are shown in Table 1. With regard to superoxide, ascorbate reacts at a similar rate compared with tetrahydrobiopterin (BH_4), but reacts 100-fold faster than estimated rates for α-tocopherol in micelles. Uric acid itself it not reactive with superoxide, although its monoradical species are quite reactive. Both BH_4 and α-tocopherol react much faster than ascorbate with peroxynitrite, whereas rates with uric acid are similar.

Although ascorbate is widely considered an antioxidant, its reduction of transition metals such as Fe^{3+} and Cu^{2+} is well known to generate reactive oxygen species, including the hydroxyl radical (25). These reactions are potentially important for interpretation of *in vitro*, cell culture, or tissue incubation experiments, where free metal ions might exist. On the other hand, an extensive review of *in vivo* studies by Carr and Frei (28) found little evidence for a pro-oxidant effect of ascorbate on markers of DNA, protein, and lipid oxidation.

Recycling of intracellular radicals of cellular constituents and enzyme co-factors

Ascorbate also recycles and, thus, preserves several readily oxidized molecules in the cell. It repairs both protein (45) and lipid radicals (55). The latter occurs indirectly as ascorbate recycling of α-tocopherol in cell membranes. When α-tocopherol reacts with lipid peroxyl radicals in the lipid phase, it is oxidized to the α-tocopheroxyl radical, which ascorbate subsequently reduces to α-tocopherol (24, 122). Ascorbate recycling of the α-tocopheroxyl radical has been observed in lipid micelles (18, 122) and likely accounts for sparing of α-tocopherol in erythrocyte ghosts (34), in intact cells (111), and in human smokers (23). This sparing of α-tocopherol by ascorbate has also been observed in endothelial cells cultured in the presence of oxidized LDL (135).

An important enzyme co-factor recycled by ascorbate is BH_4. BH_4 maintains the catalytic iron of several dioxygenase enzymes in the active ferrous form. These enzymes mediate the hydroxylation of key neurotransmitter precursors, triglyceride precursors, and the synthesis of nitric oxide (Table 2). Regarding the latter, ascorbate recycling of BH_4 is especially important for the proper function of endothelial nitric oxide synthase (eNOS) (Fig. 5) (72, 83). BH_4 is synthesized *de novo* through a pathway controlled by GTP-cyclohydrolase-1 and by recycling of dihydrobiopterin (BH_2) by dihydrofolate reductase. BH_4 (but not ascorbate) is a required co-factor for eNOS, along with arginine and molecular oxygen. During the

Table 2. Tetrahydrobiopterin-Dependent Enzymes

Enzyme	*Substrate*	*Product(s)*	*Relevance*
Phenylalanine hydroxylase	*L*-phenylalanine	*L*-tyrosine	Phenylketonuria when deficient
Tyrosine hydroxylase	*L*-tyrosine	*L*-DOPA	First and rate-limiting step in catecholamine synthesis
Tryptophan hydroxylase	*L*-tryptophan	5-hydroxy-tryptophan	First and rate-limiting step in serotonin synthesis
Nitric oxide synthase	*L*-arginine	*L*-citrulline, nitric oxide	Nitric oxide generation
Alkylglycerol monooxygenase	1-alkyl-*sn*-glycerol	1-hydroxyalkyl-*sn*-glycerol	Glycerol and lipid metabolism

enzyme cycle, BH_4 donates an electron to the active dimeric form of eNOS ($eNOS_d$) and becomes the trihydrobiopterin radical ($BH_3^{\bullet}$), which is efficiently reduced back to BH_4 by ascorbate, with a rate constant of $\sim 1.7 \times 10^5\, M \cdot s^{-1}$ (127) (Fig. 5). This reaction is very specific for ascorbate, as thiols are ineffective (83). Failure to reduce the $BH_3^{\bullet}$ results in its decomposition to inactive BH_2, which then competes with a similar affinity as BH_4 for binding on eNOS, but does not support $NO^{\bullet}$ production (36). Lack of BH_4, combined with increased BH_2, uncouples eNOS (152), causing the enzyme to generate superoxide instead of $NO^{\bullet}$. What $NO^{\bullet}$ is generated then may rapidly react with this superoxide to form peroxynitrite (13), which will both consume $NO^{\bullet}$ and oxidize more BH_4 (Table 1) (83). In this regard, any scavenging of superoxide by ascorbate will also prevent its reaction with $NO^{\bullet}$ to form peroxynitrite (148). Another consequence of uncoupled eNOS noted in Figure 5 is that it dissociates from the plasma membrane as well as from itself to form unbound monomers ($eNOS_m$) that generate superoxide (83, 89).

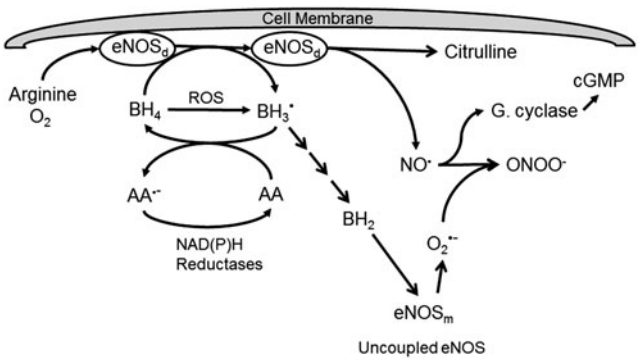

FIG. 5. Ascorbate recycling of BH_4 and preservation of nitric oxide. Dimeric eNOS ($eNOS_d$) attached to the endothelial cell plasma membrane utilizes arginine, molecular oxygen, and BH_4 to generate nitric oxide ($NO^{\bullet}$) that subsequently activates endothelial and smooth muscle guanylate cyclase (G. cyclase). In the enzyme cycle, the trihydrobiopterin radical ($BH_3^{\bullet}$) is generated, which is recycled by ascorbate (AA). The resulting ascorbate radical ($AA^{\bullet -}$) is recycled by various NAD(P)H-dependent reductases. Failure to recycle $BH_3^{\bullet}$, or its formation due to BH_4 oxidation by reactive oxygen species (ROS), results in the formation of dihydrobiopterin (BH_2), which competes with BH_4 for the enzyme. This, and loss of BH_4 uncouples eNOS, which then dissociates from the membrane into monomers ($eNOS_m$) that generate superoxide ($O_2^{\bullet -}$) rather than $NO^{\bullet}$. Reaction of $O_2^{\bullet -}$ with any available $NO^{\bullet}$ forms peroxynitrite, a strong nitrating oxidant. By initially recycling BH_4, ascorbate prevents loss of BH_4 and sustains eNOS activity. BH_4, tetrahydrobiopterin

It is evident that the ability of eNOS to generate $NO^{\bullet}$ depends both on synthesizing BH_4 and on recycling it from BH_2 through the pathways controlled by GTP-cyclohydrolase-1 and dihydrofolate reductase, respectively. Nonetheless, ascorbate recycling of the $BH_3^{\bullet}$ at a site proximal in the pathway of oxidation of BH_4 to BH_2 (Fig. 5) would also be expected to maintain effective BH_4/BH_2 ratios, thus lessening the need for recycling from BH_2 by dihydrofolate reductase.

Regulation of enzyme phosphorylation

Ascorbate was found by Wilson's group to inhibit the increased activity of protein phosphatase type 2A (PP2A) in cultured primary endothelial cells exposed to a septic insult (bacterial lipopolysaccharide [LPS] + interferon-γ [IFNγ]) (66). The proposed mechanism of this effect is depicted in the left-hand cascade in Figure 6. Stimulation of NADPH oxidase by LPS + IFNγ generates superoxide, which in endothelial cells rapidly reacts with available nitric oxide, generating peroxynitrite. The latter can then nitrate the catalytic subunit of PP2A, leading to its activation (183), which, in turn, was shown to dephosphorylate occludin and disrupt the endothelial permeability barrier (66). Ascorbate inhibits this process by scavenging both superoxide and peroxynitrite (Fig. 6). Wu, *et al.* (182) hypothesized that such scavenging by ascorbate would decrease positive feedback of reactive oxygen/nitrogen species on NADPH oxidase subunit assembly and, thus, indirectly inhibit enzyme function.

More recently, ascorbate inhibition of PP2A activity has been implicated in the regulation of eNOS in non-stimulated primary and immortalized human umbilical vein endothelial cells (85) (Fig. 6). PP2A is a key phosphatase involved in dephosphorylation of multiple protein kinases (116) and other enzymes, including eNOS (117). In the study by Ladurner, *et al.* (85), ascorbate was shown to increase phosphorylation of eNOS-Ser^{1177} and decrease phosphorylation of eNOS-Thr^{495}. This pattern of phosphorylation changes is known to substantially activate the enzyme (117). These phosphorylation effects were rapid and complete within 30 min, although the comparable increase in eNOS activity required 16 h of incubation with ascorbate. An ascorbate-dependent increase in BH_4 required 24 h, suggesting that the observed enzyme activation was not due to preservation of BH_4 by ascorbate. These investigators went on to implicate inhibition of PP2A as the mechanism of ascorbate-stimulated eNOS-Ser^{1177} phosphorylation. It is known that PP2A dephosphorylates eNOS-Ser^{1177} (117), so that its inhibition could allow kinases to increase eNOS-Ser^{1177} phosphorylation (Fig. 6). Ladurner, *et al.* (85) found that the ascorbate-dependent increase in phosphorylation of eNOS-Ser^{1177} was blocked by overexpression

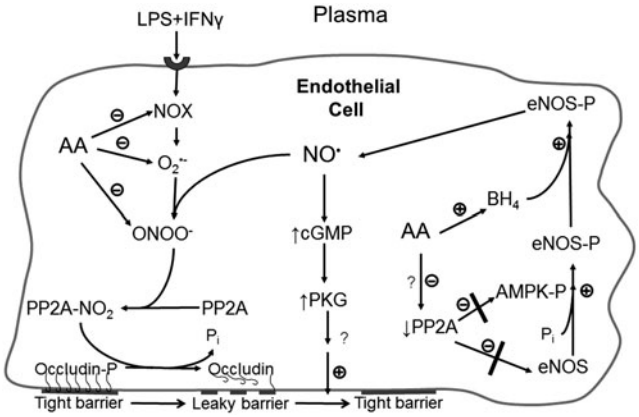

FIG. 6. Multiple mechanisms by which ascorbate preserves nitric oxide and tightens the endothelial permeability barrier. In endothelial cells in which NADPH oxidase (NOX) is activated by septic insult (or other mechanisms), the resulting superoxide ($O_2^{\bullet-}$) reacts with available nitric oxide ($NO^{\bullet}$) to form peroxynitrite ($ONOO^-$), which nitrates and activates PP2A. The phosphatase then dephosphorylates occludin, causing it to pull away from the membrane and weaken tight junctional structures. Ascorbate prevents the activation of PP2A in this pathway by inhibiting NOX function and scavenging $O_2^{\bullet-}$ and $ONOO^-$. In unstimulated cells (with presumably low levels of $ONOO^-$, ascorbate also enhances nitric oxide generation by inhibiting PP2A by an unknown mechanism. This prevents PP2A from dephosphorylating and thus deactivating eNOS itself, as well as the AMP-dependent kinase (AMPK). The resulting increase in eNOS phosphorylation is mediated at least in part by phosphorylation-dependent activation of AMPK, which activates eNOS to generate nitric oxide. This, along with the preservation of BH_4 by ascorbate, increases intracellular nitric oxide, which then generates cyclic GMP through the canonical pathway to eventually tighten the endothelial permeability barrier. PP2A, protein phosphatase type 2A.

of the catalytic subunit of PP2A and that the ascorbate-induced phosphorylation patterns of both eNOS-Ser^{1177} and eNOS-Thr^{495} were mimicked by specific inhibition of PP2A with okadaic acid. They also determined by inhibitor and knockdown experiments that the ascorbate-induced phosphorylation of eNOS-Ser^{1177} was likely due to the activation of AMP kinase (AMPK), which showed rapid and sustained increased phosphorylation at AMPK-Thr^{172} in response to ascorbate. PP2A is known to dephosphorylate AMPK on Thr^{172} (181), so that its inhibition by ascorbate could result in an increase in its phosphorylation and activation (Fig. 6). Although inhibition of PP2A could nicely explain the stimulation of eNOS activity by ascorbate, there remains the caveat that Han, *et al.* (66) did not find ascorbate to inhibit PP2A activity in unstimulated endothelial cells. This, as well as the mechanism of PP2A inhibition by ascorbate in unstimulated cells, will require further studies.

Enzyme co-factor function

Another well-established function of ascorbate is to sustain the activity of mono- and dioxygenase enzymes (5). These enzymes vary in their substrates, tissue localizations, and mechanisms (Table 3). The two monooxygenases that are dependent on ascorbate are important in catecholamine and neuropeptide synthesis. Of the more numerous dioxygenases, only 4-hydroxyphenylpyruvate hydroxylase mediates the incorporation of both atoms of dioxygen into the same molecule, whereas all the other enzymes incorporate one atom of dioxygen into α-ketoglutarate and the other into the product of the reaction (Fig. 7). The dioxygenase enzymes that hydroxylate collagen and other substrates are perhaps the best known of the group. They serve to hydroxylate proline and lysine in procollagen, thus allowing proper folding of the procollagen chain and release from the cell as mature collagen (75, 118). As with the other dioxygenases, these hydroxylases have a non-heme iron in the active site that is kept in its active, reduced form by ascorbate (39, 119, 120). Ascorbate, however, is not involved in the coupled reaction that hydroxylates the protein and converts α-ketoglutarate to succinate and CO_2. Rather, ascorbate prevents deactivation of the enzyme during an occasional uncoupled reaction cycle in which the peptide substrate fails to bind or α-ketoglutarate is cleaved directly (Fig. 7). The ferryl ion bound to the enzyme decomposes, leaving ferric iron bound to the enzyme, which is then directly reduced by ascorbate to ferrous iron and thus able to enter a new reaction cycle. The resulting ascorbate radical is then recycled by mechanisms described earlier.

Although the mechanism by which ascorbate stimulates collagen synthesis is often considered to involve procollagen hydroxylation, many studies suggest that the major effect of ascorbate is to stimulate *de novo* collagen synthesis. For example, scorbutic guinea pigs had no differences in urinary hydroxyproline excretion (9) and only about a 10% decrease in skin collagen synthesis (10) compared with non-scorbutic guinea pigs. These findings are in agreement with more recent studies of mice lacking the SVCT2 transporter. Despite very low tissue levels of ascorbate in these mice, skin hydroxyproline content was normal (146). On the other hand, moderate vitamin C deficiency in guinea pigs caused substantial decreases in type IV collagen mRNA in vessel walls (94). Addition of ascorbate increases procollagen mRNA in cultured fibroblasts (58, 118) and stimulates type IV collagen maturation and release in a concentration-dependent manner in cultured endothelial cells (96, 103, 131, 184). As reviewed by Englard and Seifter (51), although many studies report contradictory evidence, both pre- and post-translational mechanisms are likely to be involved in ascorbate-stimulated deposition of mature collagen in the vessel wall and integument.

A second Fe^{2+}-dependent prolyl hydroxylase isoform that requires ascorbate is the enzyme that hydroxylates the hypoxia-inducible transcription factor subunit HIF-1α. Hydroxylation of the oxygen-sensitive HIF-1α subunit on specific proline or lysine residues targets it for ubiquitination and proteosomal destruction (29). Lack of ascorbate prevents this hydroxylation and allows HIF-1α to accumulate (21, 63). The surviving HIF-1α then forms a nuclear transcription complex that activates diverse pathways, including those for glucose transport and metabolism, angiogenesis, inflammation, and cell survival (22, 68, 168). In both primary culture (167) and immortalized (130) human umbilical vein endothelial cells, provision of ascorbate abolished basal HIF-1α expression and decreased the HIF-1α expression stimulated by culture of cells under hypoxic conditions or in the presence of cobalt.

Recent studies have also suggested that ascorbate can affect gene expression by serving as a co-factor for demethylases of both DNA and histones. Similar to the prolyl/lysyl

Table 3. Molecular Functions of Ascorbate

Function	*Substrate*	*Product(s)*	*Relevance*
Primary antioxidant	^{-}OH, $O_2^{\bullet -}$, $RO^{\bullet}$	H_2O, H_2O_2, ROH	Scavenges damaging radicals (24)
Pro-oxidant	Fe^{3+}, O_2	Fe^{2+}, H_2O_2	Redox regulation (140), DNA, or protein damage (25, 65)
Recycling			
Tetrahydrobiopterin	$BH_3^{\bullet}$	BH_4	Numerous, see Table 2
Lipid peroxidation	α-Tocopheroxyl radical	α-Tocopherol	Chain breaking of lipid peroxidation (79, 122)
Monoxygenases			
Dopamine β-hydroxylase	Dopamine	Norepinephrine	Catecholamine biosynthesis (113, 149)
Peptidylglycine α-amidating monoxygenase	Neuroendocrine peptides	α-Amidated peptides	Neuropeptide and neurotransmitter synthesis (50)
Dioxygenases			
4-Hydroxyphenylpyruvate hydroxylase	4-Hydroxyphenyl-pyruvate	Homogentisate	Breakdown of *L*-tyrosine (88)
Prolyl/lysyl hydroxylase (collagen, elastin)	α-KG, peptide	Peptide-OH	Collagen, elastin synthesis (10, 118)
Prolyl/lysyl hydroxylase (HIF-1α)	α-KG, HIF-1α	Hydroxylated HIF-1α	Proteosomal degradation of HIF-1α (21, 63)
Thymine/pyrimidine hydroxylases	α-KG, thymine, deoxyuridine	Hydroxylated nucleobases	Salvage pathways (51)
6-*N*-Trimethyl-*L*-lysine hydroxylase	α-KG, 6-*N*-trimethyl-*L*-lysine	3-hydroxy-N^{ε}-trimethyllysine	1st step in carnitine biosynthesis (121, 132)
γ-Butyrobetaine hydroxylase	α-KG, γ-butyrobetaine	L-carnitine	Last step in carnitine biosynthesis (121, 132)
Histone demethylase Jhdm1a/1b	α-KG, trimethylated histone H3	Mono-methylated histone H3	Epigenomic regulation, somatic stem cell reprogramming (74, 171)
Nucleic acid demethylase	α-KG, 3-methyl-thymine in DNA	Thymine in DNA	Reprogramming of fibroblasts to induced pluripotent stem cells (32)

HIF-1α, hypoxia inducible factor-1α; Jhdm1a/1b, Jumonji histone demethylase 1a/1b; α-KG, α-ketoglutarate; R, protein or lipid carbon.

hydroxylases, these demethylases are Fe^{2+}-α-ketoglutarate dioxygenases, having a non-heme active site iron that is maintained in the ferrous state by ascorbate. The DNA demethylases remove methyl groups from thymine residues (59), which may, in turn, activate a variety of genes (32). Activation of these demethylases by ascorbate could account for the widespread DNA demethylation observed in response to ascorbate treatment of human embryonic stem cells (32) and could promote their differentiation (33). The histone demethylases having Jumonji C catalytic domains remove methyl groups from trimethylated lysines on DNA-bound histones, thus opening up the DNA for potential transcription (160). These enzymes may also promote widespread histone demethylation in response to ascorbate, as well as enhance the ability of the vitamin to remove the senescence block on mouse and human induced pluripotent stem cells (52, 171) and facilitate T-cell maturation (Manning, *et al.*, this Forum).

Role of Ascorbate in Endothelial Function

Ascorbate effects on endothelial cell proliferation and apoptosis

Ascorbate has several effects on endothelial function and survival. It causes endothelial cells to proliferate and to form capillary-like structures in cell in culture (136, 139). It is likely that the effect of ascorbate to stimulate endothelial cell proliferation is due to its ability to increase the synthesis of type IV collagen (143, 157), as in the absence of ascorbate there is little generation of mature type IV collagen by cultured endothelial cells (184) or of type IV collagen mRNA relative to that of elastin in blood vessels (94). Further, it has been shown that type IV collagen is required for both basement membrane formation (114) and endothelial cell adhesion (133), processes that are not facilitated by collagen types I and III (133).

Ascorbate also prevents apoptosis of endothelial cells in culture induced by high glucose conditions (73, 154), tumor necrosis factor-α (136), and LPS (64). In humans with congestive heart failure, an intravenous bolus of 2.5 g of ascorbate followed by 3 days of 2 g vitamin C a day decreased plasma levels of circulating endothelial apoptotic microparticles to 32% of baseline levels, an effect also significant compared with a placebo-treated control group of the same clinical characteristics (134).

Although the foregoing evidence favors a positive effect of ascorbate on endothelial growth and survival, ascorbate has also been noted as an angiostatic factor, inhibiting the formation of capillary-like structures in cultured endothelial cells and decreasing *in vivo* angiogenesis in chick chorioallantoic

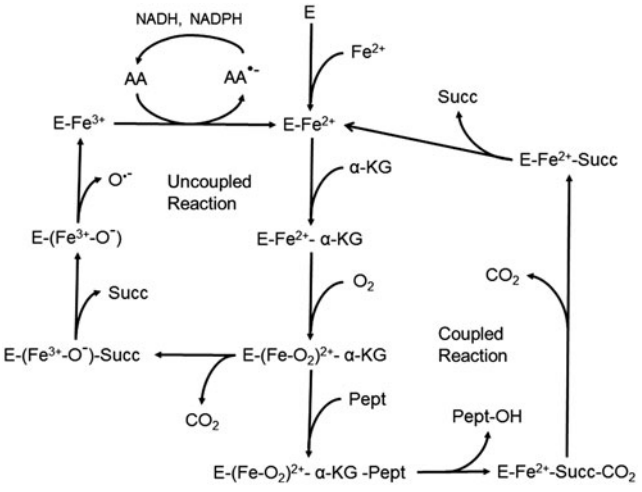

FIG. 7. Role of ascorbate in maintaining the activity of α-ketoglutarate-dependent hydroxylation reactions. The proposed mechanism of α-ketoglutarate-dependent hydroxylases is first a sequential binding of ferrous iron, α-ketoglutarate, and molecular oxygen. At this point, there is the formation of a key ferryl-oxygen intermediate that not only usually binds the substrate protein (Pept), but can also generate an uncoupled cycle by cleaving α-ketoglutarate to succinate (Succ) and releasing CO_2. In the functional enzyme cycle, a proline or lysine is hydroxylated with subsequent sequential release of the modified protein, CO_2, and succinate leaving enzyme-bound ferrous iron that can restart the cycle. In an uncoupled cycle, the enzyme-bound iron is oxidized to ferric iron by oxygen, and the latter is released, perhaps as the hydroxyl radical ($O^{\bullet -}$). The function of ascorbate is to reduce the enzyme-bound ferric to ferrous iron, thus allowing the enzyme to enter a potentially functional cycle again.

membranes (6). It is likely, however, that such effects are related to the use of very high ascorbate concentrations, which have been shown to inhibit angiogenesis in excised aortic rings and in subcutaneous Matrigel plugs *in vivo* (115). In addition, ascorbate deficiency in mice unable to synthesize vitamin C markedly impaired angiogenesis in a transplanted tumor model (156). Although ascorbate deficiency clearly impairs endothelial proliferation and angiogenesis, the effects of ascorbate may differ with the amount of ascorbate available.

Ascorbate modulation of vascular tone

Deficiency of nitric oxide causes endothelial dysfunction that manifests as failure of arterioles to dilate in response to stimuli such as shear stress or acetylcholine. As previously noted, ascorbate spares endothelial cell-derived nitric oxide by recycling BH_4 (Fig. 5) and by inhibiting the function of PP2A (Fig. 6). It may also preserve nitric oxide by several other mechanisms, including direct reduction of nitrite to nitric oxide, release of nitric oxide from nitrosothiols, and, as noted earlier, by scavenging superoxide that would otherwise react with nitric oxide to form peroxynitrite [reviewed in (97)]. These mechanisms may combine to generate more nitric oxide, which then diffuses out of endothelial cells and binds to an iron coordinate site on the active site heme of guanylate cyclase in adjacent smooth muscle cells (26). This activates the enzyme and increases production of cyclic GMP, causing the smooth muscle cells to relax and the vessel to dilate (Fig. 5). Nitric oxide also inhibits the toxicity of pro-inflammatory cytokines and adhesion molecules that are responsible for endothelial dysfunction and ultimately, atherosclerosis (38, 81, 82).

The effects of ascorbate to promote endothelial- and nitric oxide-dependent vasodilation have also been confirmed in clinical studies of endothelium-dependent vasodilatation in patients with endothelial dysfunction due to atherosclerosis (60, 71, 159), hypertension (48, 49, 153), smoking (70, 80, 145), and diabetes (158). Most of these studies used high doses of intravenous ascorbate, which is often infused directly into coronary arteries. However, it has been shown in a randomized, double-blind, placebo-controlled study that long-term moderate oral ascorbate supplements improved endothelial-dependent flow-mediated brachial artery dilation in people with atherosclerosis (60). In this study, 46 subjects with coronary artery disease were administered 500 mg/day of ascorbate orally for 30 days. The results showed that the already normal plasma ascorbate concentrations were doubled by the supplement and that flow-mediated brachial artery dilation increased by 50%. Although it has not been possible to show that ascorbate or related antioxidants decrease event frequency in established atherosclerosis (69, 142), these results suggest that ascorbate might prevent or delay the early endothelial dysfunction that is associated with atherosclerosis.

Although ascorbate facilitates nitric oxide-dependent vasodilation, it impairs tonic vasodilation due to endothelial-derived hyperpolarizing factor (EDHF) (112, 150). Despite considerable effort, the identity of EDHF has not been ascertained (151). Ascorbate inhibits both flow- and agonist-mediated vasodilatation due to EDHF, the latter probably due to an antioxidant effect, as it is inhibited by thiols and reducing agents (112).

Ascorbate-stimulated tightening of the endothelial permeability barrier

Ascorbate has been shown to tighten the permeability barrier of endothelial cells in long-term culture on porous filter supports (163). In those studies, bovine arterial endothelial cells were cultured for 5–7 days, and daily addition of (10–100 μM) ascorbate progressively decreased transfer of fluorescein dextran across the cells and filter barriers. Inhibitors of collagen synthesis prevented ascorbate-dependent barrier tightening, leading the authors to conclude that the effect required collagen synthesis. Subsequent studies carried out at much shorter times of culture with ascorbate (90 min or less) found that intracellular ascorbate acutely tightened the endothelial barrier to its own transit in both EA.hy926 cells (an immortalized line derived from human umbilical vein endothelial cells) and primary culture dermal microcapillary endothelial cells (108). The tightening in EA.hy926 cells was significant after 15 min of treatment with dehydroascorbate (used to rapidly load them with ascorbate) and paralleled the intracellular ascorbate concentration. The effect of ascorbate persisted with time in culture, as a single addition of 30–300 μM ascorbate caused progressive 30%–70% decreases in ascorbate transfer when performed after 18 h in culture. In these experiments, removal of the cells from the filters and matrix had no effect on ascorbate permeability, indicating that the effect was not due to collagen deposition (108). Although

there were no major changes in F-actin configuration in the EA.hy926 cells, inhibition of microtubule function with colchicine prevented the effect of ascorbate to inhibit its own transfer (108).

Intracellular ascorbate also decreased transfer of radiolabeled inulin (5000–5500 kDa) and mannitol across the EA.hy926-generated endothelial barrier, showing that the effect was not limited to ascorbate transfer alone (108). Loading endothelial cells with ascorbate also prevented the increases in barrier permeability caused by thrombin and high glucose concentrations (J.M. May, unpublished observations). Finally, ascorbate both prevented and reversed the effect of oxidized LDL to increase endothelial barrier permeability, an effect that was mimicked by several other antioxidants (105). Although this might suggest a simple antioxidant effect, a subsequent study showed that the ascorbate-induced decrease in radiolabeled inulin permeabilty in EA.hy926 cells was blocked either by inhibiting the function of eNOS with N_{ω}-nitro-*L*-arginine methyl ester (*L*-NAME) or by inhibiting the effect of nitric oxide to activate guanylate cyclase (106). Nitric oxide derived from eNOS has been shown to decrease both basal (128) and stimulated (46) endothelial permeability, an effect that is dependent on the generation of cyclic GMP (46, 175). Although details of the mechanism by which ascorbate might tighten endothelial permeabilty by the nitric oxide-cyclic GMP pathway remain to be determined, the mechanisms depicted in Figure 6 support a coordinated function for ascorbate-induced increases in nitric oxide in decreasing both basal and agonist-stimulated permeability.

Conclusion and Clinical Relevance

The foregoing section generally describes a beneficial role for ascorbate in endothelial growth, survival, and function to maintain vascular responsiveness and integrity. Due to the kinetics of the SVCT2, ascorbate concentrations in human white blood cells and platelets saturate at high normal plasma ascorbate concentrations. The observation that cultured endothelial cells expressing the SVCT2 saturate over a similar concentration range of ascorbate suggests that they have similar low millimolar ascorbate concentrations *in vivo*, which also cannot be increased even by substantial increases in dietary intake. This, and the fact that most intracellular functions of ascorbate would be saturated as well, suggests that megadoses of vitamin C supplements will not be beneficial when normal intakes are much more than 200 mg a day. As reviewed and commented on (1, 125), this likely is a contributing factor to the fact that supplements of ascorbate and other antioxidants to participants in large clinical trials failed to show benefit on atherosclerosis progression (35, 69, 142). Indeed, in most clinical trials, plasma ascorbate levels were not measured, making it possible to say that the trials could have failed because ascorbate levels were not significantly increased. Other confounding factors could be combined use of several antioxidants (69) or a high rate of statin use in the trials (166). Although there is strong preclinical and clinical evidence to support an effect of ascorbate on endothelial dysfunction in early atherosclerosis, clinical trials thus far have studied subjects either with established atherosclerosis or with a high risk of disease. Unfortunately, given the size and duration that would be required for a primary prevention trial, it seems unlikely that one will ever be carried out.

More revealing may be to study the numerous conditions and diseases in which ascorbate is depleted, some of which are clearly known to have endothelial dysfunction as a prelude to vascular dysregulation, leakage, or atherosclerosis. These conditions/diseases include metabolic syndrome (54), diabetes with poor glycemic control (129), atherosclerosis (86), smoking (92, 93, 138), poor dietary intake (7, 27), inflammatory neurodegenerative diseases (129), sepsis (31, 44, 169), and hemodialysis (170, 185). Indeed, it may even require megadoses of the vitamin to replete the vitamin and improve endothelial function in some of these conditions. For example, taking 800 mg a day of oral vitamin C for 4 weeks doubled low vitamin C plasma levels in subjects with insulin-resistant type 2 diabetes, but not to levels expected from this dose of the vitamin. Moreover, this treatment did not improve forearm blood flow in response to acetylcholine or insulin resistance. A similar difficulty in repleting low vitamin C levels was found in critically injured or infected patients, even with intravenous infusions of approximtaely 1000 mg of vitamin C a day (90).

Supplementation to upper normal plasma ascorbate levels is clearly indicated in most diseases and conditions in which ascorbate is depleted. However, it is seldom a priority, because patients, physicians, and health authorities are unaware of the increasing evidence for multiple potentially important functions of ascorbate. With regard to the endothelium, it is worth emphasizing observations made more than 50 years ago that early scurvy generates endothelial disruption in guinea pigs, which resembles atherosclerosis (176, 177) and is fully and rapidly reversible with ascorbate repletion (177).

Acknowledgment

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Abbreviations Used

AMPK = AMP kinase
BH_2 = dihydrobiopterin
$BH_3^{\bullet}$ = trihydrobiopterin radical
BH_4 = tetrahydrobiopterin
DHA = dehydroascorbate
EDHF = endothelial-derived hyperpolarizing factor
eNOS = endothelial nitric oxide synthase
HIF-1α = hypoxia-inducible factor-1α
IFNγ = interferon-γ
LDL = low density lipoprotein
LPS = lipopolysaccharide
PP2A = protein phosphatase type 2A
SVCT1 = sodium-dependent vitamin C transporter 1
SVCT2 = sodium-dependent vitamin C transporter 2