



Does vitamin C enhance nitric oxide bioavailability in a tetrahydrobiopterin-dependent manner?

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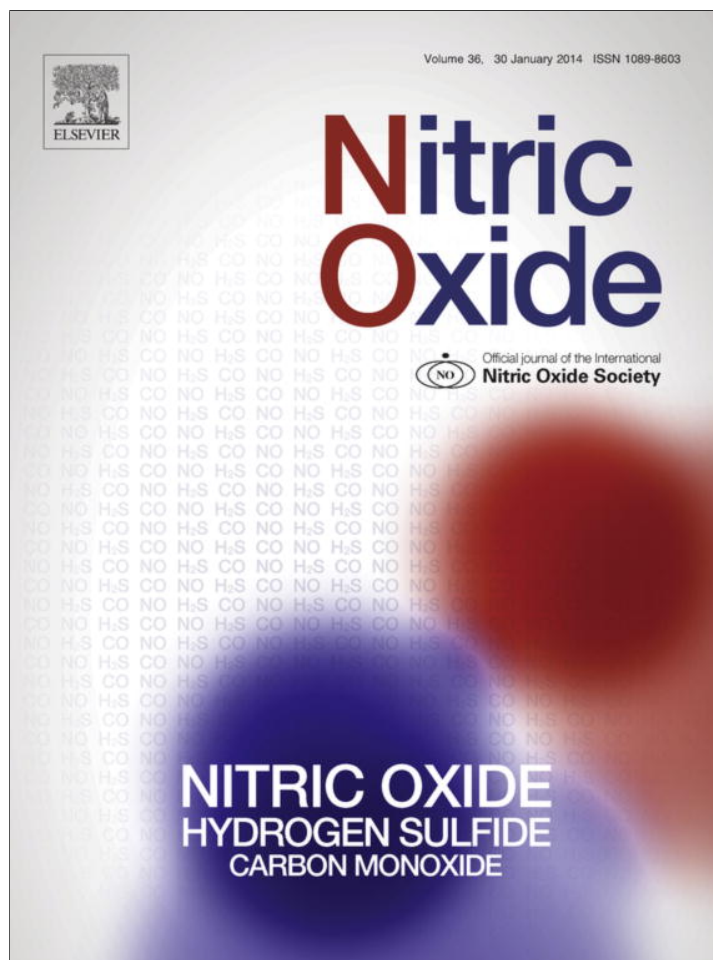
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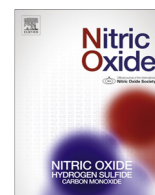
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Review

Does vitamin C enhance nitric oxide bioavailability in a tetrahydrobiopterin-dependent manner? *In vitro*, *in vivo* and clinical studies



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ABSTRACT

Ascorbate (Asc) has been shown to increase nitric oxide (NO) bioavailability and thereby improve endothelial function in patients showing signs of endothelial dysfunction. Tetrahydrobiopterin (BH₄) is a co-factor of endothelial nitric oxide synthase (eNOS) which may easily become oxidized to the inactive form dihydrobiopterin (BH₂). Asc may increase NO bioavailability by a number of mechanisms involving BH₄ and eNOS. Asc increases BH₄ bioavailability by either reducing oxidized BH₄ or preventing BH₄ from becoming oxidized in the first place. Asc could also increase NO bioavailability in a BH₄-independent manner by increasing eNOS activity by changing its phosphorylation and S-nitrosylation status or by upregulating eNOS expression. In this review, we discuss the putative mechanisms by which Asc may increase NO bioavailability through its interactions with BH₄ and eNOS.

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Introduction

Tetrahydrobiopterin (Fig. 1) is an endogenous co-factor of eight human enzymes: four aromatic amino acid hydroxylases, three nitric oxide synthases and alkylglycerol monooxygenase [1]. The

three nitric oxide synthases (NOS) are neuronal NOS (nNOS or NOS1), inducible NOS (iNOS or NOS2) and endothelial NOS (eNOS or NOS3). NOS produces the vaso-relaxing factor nitric oxide (NO) in a process involving conversion of L-arginine to L-citrulline, and tetrahydrobiopterin is thus important for regulating blood pressure. The active form of NOS producing NO is dimeric and one of the roles of tetrahydrobiopterin is to stabilize this dimer [2].

Biopterin exists in three stable oxidation states: tetrahydrobiopterin (BH₄), dihydrobiopterin (BH₂) and biopterin. Besides these,

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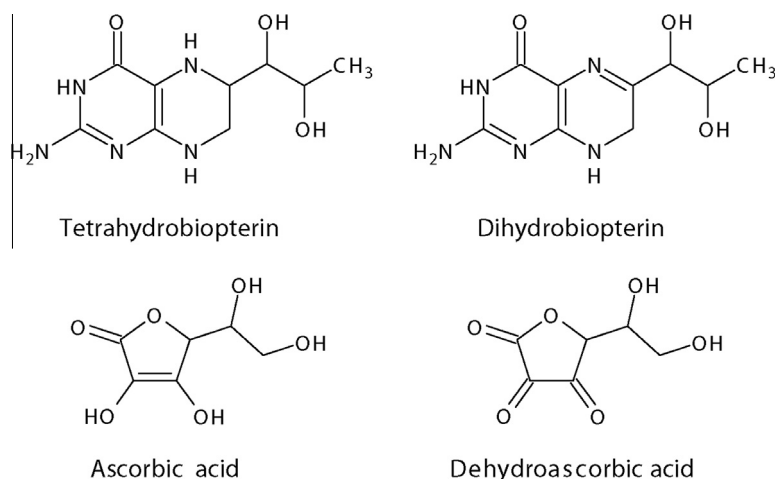


Fig. 1. Structures of tetrahydrobiopterin, dihydrobiopterin, ascorbic acid and dehydroascorbic acid.

unstable short-lived intermediates such as the trihydrobiopterin radical and quinoid dihydrobiopterin exist. The most reduced form, BH₄, is easily oxidized to a quinoid form of BH₂; qBH₂, which rapidly rearranges to BH₂; BH₂ may then be further oxidized to biopterin. However, in contrast to BH₄, BH₂ is relatively stable and is only slowly oxidized [3].

BH₄ and BH₂ have been shown to bind to eNOS with similar affinity ($K_d \approx 80$ nM) [4]. However, while binding of BH₄ to NOS leads to formation of NO, binding of BH₂ completely inhibits the formation of NO [5] and instead leads to formation of superoxide [6]; a NOS state referred to as uncoupled. Because of this competition for binding to NOS, it could be expected that it is the ratio of BH₄-to-BH₂ that is important in determining superoxide and NO production, and hence endothelial dysfunction, rather than the absolute amount of BH₄. Indeed, in a number of studies this association has been observed.

Ascorbate (Asc), the reduced form of vitamin C, is an important intracellular and circulatory antioxidant and has been suggested to play an important role in maintaining BH₄ in its reduced state. Thus, *in vitro* studies have shown that ascorbate selectively reduces the BH₃· radical to BH₄, i.e., the one-electron oxidation product [7,8]. Also, Asc have been suggested to non-selectively spare BH₄ from oxidation through its generic antioxidant function [8]. In this way, Asc may indirectly improve vascular function by increasing the bioavailability of NO *in vivo*. In these processes, Asc itself is oxidized to the ascorbyl free radical, two molecules of which dismutate into one molecule of Asc and one of dehydroascorbic acid

(DHA), the fully oxidized form of vitamin C (Fig. 2). In order to prevent loss of vitamin C through oxidation, dehydroascorbic acid is rapidly reduced to Asc intracellularly by e.g., glutathione or thioredoxin reductase in a number of cell types – so called ascorbate recycling [9]. An intimate relationship between Asc, BH₄ and NO production would provide a rationale partly explaining the significant relationship between poor vitamin C status and cardiovascular disease risk that has consistently been reported from large epidemiological studies [10]. However, until now *in vivo* and clinical studies investigating this role of Asc have been quite few.

This review discusses the available experimental literature on the putative role of Asc in NO bioavailability through interactions with BH₄ and NOS.

Biopterin oxidation status as biomarker of endothelial function

A number of studies have pointed out that the ratio of BH₄-to-BH₂ may be a better biomarker of superoxide and nitric oxide production, and hence endothelial function, than absolute levels of BH₄. Crabtree and co-workers found that NO bioactivity correlated with intracellular BH₄-to-BH₂ ratio and not absolute levels of BH₄, and that superoxide production was negatively correlated with BH₄-to-BH₂ ratio [4]. Flow-mediated vasodilation was found to be positively correlated with BH₄ plasma levels and negatively correlated with BH₂ plasma levels in patients with cardiovascular disorders; hence, a very strong positive correlation between flow-

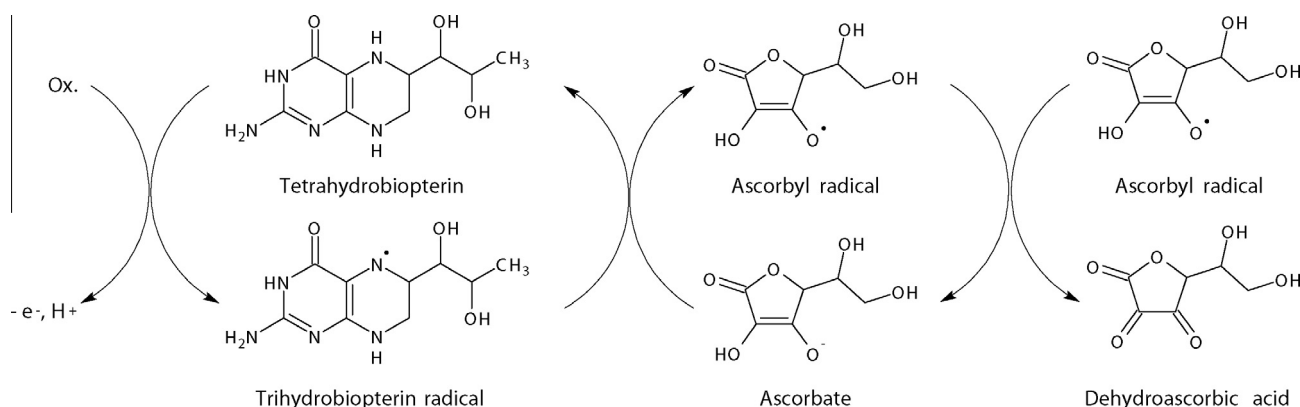


Fig. 2. Oxidation of tetrahydrobiopterin to the trihydrobiopterin radical followed by reduction by ascorbate with concomitant formation of the ascorbyl radical, two of which can dismutate yielding ascorbate and dihydroascorbic acid.

mediated vasodilation and BH₄-to-BH₂ ratio was observed [11]. Likewise, in rats treated to increase the amount of BH₂ and hence lower the BH₄-to-BH₂ ratio, evidence of uncoupling of NOS leading to increased superoxide production was found [12]. Also, acetylcholine-induced vasorelaxation was reduced in the aorta of rats with lower BH₄-to-BH₂ ratio and mean blood pressure was found to be negatively correlated with aortic BH₄-to-BH₂ ratio [12]. In another study, it was found that both BH₄ and BH₂ increased in rats that had undergone arteriovenous fistula (AVF) surgery compared to sham operated rats [13]. Despite the higher BH₄ levels in AVF rats, superoxide production was increased in AVF rats and this was attributed to a lower BH₄-to-BH₂ ratio in these rats [13]. In ApoE-deficient mice, Asc in the feed decreased the aortic level of BH₂ without influencing the level of BH₄ resulting in an increased BH₄-to-BH₂ level which correlated with an increase in NOS activity [14]. Finally, computational studies confirmed that the ratio of BH₄-to-BH₂ is important for the formation of NO and superoxide from NOS [15].

In vitro studies

As BH₄ is easily oxidized, it may easily be envisaged that an antioxidant—such as vitamin C—may enhance the stability of BH₄ and maintain a high ratio of BH₄-to-BH₂. As expected, Asc has been shown in several *in vitro* studies to be able to stabilize BH₄. First of all, the rate of autoxidation of BH₄ is dependent on the concentration of BH₄, at least at low pH, which is important to bear in mind when comparing stability studies performed at different concentrations. Thus, 41 nM BH₄ was completely converted to BH₂ in 15 min at 4 °C in 0.1 M HCl, whereas BH₄ in a 415 nM solution under the same conditions was only 1% degraded and at higher concentrations of BH₄ an even smaller percentage was oxidized [16]. Secondly, the stability of BH₄ in aqueous solution is dependent on pH showing increased stability at low pH [3]. In Table 1 is summarized the observed effects of Asc on the stability of BH₄ *in vitro*. From these studies it seems that the protective effect of Asc is modest at pH 7.4.

The question is whether the protective effect of Asc is due to Asc being able to reduce oxidized BH₄ or because Asc scavenges reactive oxygen species (ROS) before they react with BH₄; or perhaps both. It is known that Asc is not capable of reducing BH₂ [17]. It has been suggested that Asc could reduce qBH₂, the short-lived intermediate in the oxidation of BH₄, though there was no direct evidence for this mechanism [18–20]. Later on, it was suggested that thiols may reduce qBH₂, while Asc reduces one-electron oxidized BH₄ (BH₃) [21]. Direct evidence for the reduction of BH₃ by Asc has been obtained by pulse radiolysis [8] and EPR [7] and a rate constant of $1.7 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ was determined [8].

Whereas an antioxidant activity may easily be demonstrated *in vitro*, it is much more difficult to prove that the reaction is also relevant *in vivo*. BH₄ bound to NOS does become oxidized to BH₃ during the catalytic cycle of NOS [22]. However, reduction of BH₃ to BH₄ is performed by NOS and does not involve Asc [22]. Whether freely circulating BH₄ once oxidized is actually reduced by Asc *in vivo* remains to be established.

The second possibility that Asc protects BH₄ from oxidation by scavenging ROS has also been explored. The ROS of particular relevance are superoxide, as it is formed by NOS when uncoupled, and peroxynitrite as it is formed by reaction between NO, the product of NOS, and superoxide. Superoxide reacts rather slowly with both ascorbate and BH₄ with a rate constant of $3\text{--}4 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ [17,23]. Superoxide, on the other hand, reacts rapidly with NO with a rate constant of the order of $10^9\text{--}10^{10} \text{ M}^{-1} \text{ s}^{-1}$ [24–26] to produce peroxynitrite. Peroxynitrite has been shown to react 6–10 times faster with BH₄ than with Asc [7]. Thus, in order for Asc to protect BH₄ from oxidation by peroxynitrite *in vivo*, Asc must be present at a much higher concentration than BH₄. The micromolar concentration of Asc in plasma should be able to protect the nanomolar concentration of BH₄ from oxidation by these two ROS. Inside endothelial cells the concentration of BH₄ has been determined to be a few pmol/mg protein [20,27,28]. Again, the much higher intracellular concentration of Asc compared to BH₄—about three orders of magnitude [29–31]—means that Asc could kinetically protect BH₄, but other systems such as superoxide dismutase may play a more prominent role *in vivo*.

Ex vivo studies

Concerning the interaction between Asc and BH₄, three types of *ex vivo* studies have been performed: (1) studies involving NOS isolated from tissue, (2) studies involving cell cultures and (3) studies involving isolated tissues or organs. The primary focus of these studies has been to elucidate the mechanisms by which Asc may increase the bioavailability of NO.

Nos

Asc was found to increase the activity of iNOS as measured by the formation of L-citrulline, whereas dehydroascorbic acid at concentrations higher than 1 mM decreased the activity of iNOS [32]. It was suggested that this was due to an antioxidant effect of Asc, in line with the finding that Asc also decreased the apparent S_{0.5} value (the concentration at which the enzyme has half its maximal activity) of BH₄ [32], i.e., less BH₄ is needed if it's not becoming oxidized. Similarly, another study found that Asc increased the activity of eNOS in a concentration-dependent manner, as measured by

Table 1
Effect of Asc on the stability of BH₄ *in vitro*.

BH ₄ concentration (μM)	Asc concentration (μM)	Observation	Conditions	Reference
32.13	0	88% Breakdown	20 h, 4 °C, 0.1 M HCl	[16]
32.13	5700	0% Breakdown	20 h, 4 °C, 0.1 M HCl, Ar	
0.0415	5700	0% Breakdown	4 h, 4 °C, 0.1 M HCl, Ar	
0.1	0	100% Breakdown	20 min, RT, pH 7.4	[20]
0.1	100	Half-life around 70 min	RT, pH 7.4	
25	0	53% Breakdown	1 h, 22 °C, pH 7.4	[18]
25	500	46% Breakdown	1 h, 22 °C, pH 7.4	
25	1500	35% Breakdown	1 h, 22 °C, pH 7.4	
25	3000	7% Breakdown	1 h, 22 °C, pH 7.4	
20	0	100% Breakdown	0 h, 37 °C, pH 7.4	[38]
20	500	60% Breakdown	21 h, 37 °C, pH 7.4	
36	0	Half-life 17.5 min	0.5 mM EDTA, 26 °C, pH 7.4	[19]
36	100	Half-life around 25 min	0.5 mM EDTA, 26 °C, pH 7.4	

RT: room temperature.

the formation of L-citrulline [28]. BH₄ was also found to increase the activity of eNOS, as measured by the formation of L-citrulline, in microsomes prepared from human placental tissue; here, Asc enhanced the effect of BH₄ on eNOS by an additional 40% [18].

Cells

The cell types that have been studied are primarily endothelial cells but other cell types have been studied as well. Several studies have found that Asc enhanced the activity of NOS in intact cells. Increased formation of L-citrulline in the presence of Asc is often used as a biomarker of enhanced NOS activity [20,28,33–37]. Other ways of assessing the activity of NOS in the presence of Asc are formation of nitrite and nitrate, oxidation products of NO [28,34,38,39], direct detection of NO [37] or cGMP, the latter formed by action of NO on soluble guanylate cyclase [33].

Several studies have found that Asc dose-dependently increased NO bioavailability and BH₄ levels in endothelial cells (Table 2). It was found that Asc did not induce NOS expression in endothelial cells [28,33,35,39] or in macrophages [38]. Likewise, it was found that Asc did not induce increased biosynthesis of BH₄ [20,38] nor did Asc change the affinity of NOS towards BH₄ [20]. The increase in BH₄ was hence ascribed 'a stabilizing effect' of Asc on BH₄ [20]. Also, Asc did not enhance the cellular uptake of L-arginine [33], the substrate of NOS, nor did it change the K_m of L-arginine [28].

An increase in intracellular BH₄ levels increased NO bioavailability [20,28,35]. The observed increase in BH₄ levels induced by Asc may hence explain the increased NO bioavailability caused by Asc (Table 2). In addition to this effect of Asc, another mechanism has been suggested that enhances NO production without increasing BH₄ levels. It was found that Asc increased phosphorylation of eNOS-Ser¹¹⁷⁷ and decreased phosphorylation of eNOS-Thr⁴⁹⁵ and this correlated with an increased eNOS activity [36]. Intracellular BH₄ levels did not increase until after four hours, but the increase in eNOS activity was seen already after 2–4 h incubation with Asc, and it was concluded that Asc may rapidly increase eNOS activity by phosphorylation/dephosphorylation and long-term by increasing BH₄ levels [36].

Another potential mechanism involving eNOS activity has been proposed. Just like phosphorylation controls eNOS activity, so does S-nitrosylation. In a study, it was shown that S-nitrosylation inhibits eNOS activity in endothelial cells, and that eNOS S-nitrosylation correlated inversely with eNOS-Ser¹¹⁷⁷ phosphorylation [40]. Incubation of purified eNOS with Asc enhanced eNOS activity by 20% which correlated with a lower degree of eNOS S-nitrosylation [40] – the effect could not have been due to changes in phosphorylation status, as this requires the presence of phosphorylating enzymes [36]. Unfortunately, the effect of Asc on BH₄ levels was not examined in this study.

Besides endothelial cells, the effect of Asc on NOS activity has been studied in cells of the immune system. In contrast to endothelial cells, which contain eNOS, cells of the immune system contain iNOS and nNOS [37]. Neutrophils from guinea pigs on an ascorbic acid-deficient diet for two weeks were found to contain less Asc and BH₄ and have lower eNOS activity compared to neutrophils from control animals [37]. The expression of iNOS and nNOS was lower in neutrophils from Asc-deficient guinea pigs [37], whereas in endothelial cells, Asc did not influence eNOS expression (see above). Another study did not observe any changes in iNOS expression in mouse macrophage RAW 264.7 cells incubated with Asc, in contrast to a previous study that found a 2-fold increase in iNOS levels in mouse macrophage J774.1 cells [41], but did find an increase in BH₄ level and NOS activity [38]. Likewise, Asc in aqueous humor was found to increase nitrite formation in mice macrophages, an effect that was ascribed to a stabilizing effect on BH₄ observed in the growth medium [42]. In polymorphonuclear leukocytes, both Asc and DHA were found to increase NOS activity [34].

Tissues and organs

A couple of studies have looked at the effect of Asc and BH₄ on isolated tissues and organs. In excised aortas from rats, in which NOS had been inhibited *in vivo* by N^ω-nitro-L-arginine, sepiapterin, a precursor of BH₄, was found to reduce vasodilation in contrast to what might be expected. The reduced vasodilation was believed to be due to autooxidation of BH₄ caused by increased oxidative stress [43]. Incubation of the aortic rings with Asc (1 μM) for 10 min restored vasodilation and this was suggested to be due to Asc stabilizing BH₄ [43]. In another study, ascorbic acid (10 g/kg chow) was given to wild-type and ApoE-deficient mice for 26–28 weeks. Whereas Asc increased acetylcholine-induced vasorelaxation in ApoE-deficient mice, it reduced vasorelaxation in wild-type mice [14]. In aortic homogenate, Asc in the diet increased L-citrulline formation in both wild-type and ApoE-deficient mice without affecting NOS expression, in line with the findings in endothelial cells (see above). Again, protection of BH₄ by Asc was suggested to explain the findings [14]. The activity of eNOS and nNOS increased while iNOS activity decreased in ischemic rat muscle homogenate from rats fed BH₄, L-arginine and Asc (alone or in combination) with the largest change seen in the BH₄ + L-arginine + Asc group [44]. This correlated with an increased eNOS expression and NO production found *in vivo* (see below). Okazaki et al. induced diabetes in rats and their hearts were isolated and perfused. BH₄ and the BH₄-to-BH₂ ratio were decreased in the diabetic hearts whereas superoxide increased. Treatment with Asc increased the BH₄-to-BH₂ ratio, decreased superoxide production and increased nitrite and nitrate [45].

Table 2
Effect of Asc on eNOS activity and intracellular BH₄ levels in endothelial cells.

Cells	Extracellular Asc (μM)	Intracellular Asc pmol/mg protein	Effect on BH ₄	Effect on NO bioavailability	Reference
HUVEC	0.1–100	21,500	n.d.	3-Fold increase in L-citrulline and cGMP	[33]
PAEC	75	20,000	Increased 126%	59–73% Increase in cGMP, L-citrulline and nitrite/nitrate	[28]
HUVEC	1–1000	n.d.	3-Fold increase	Dose-dependent increase of L-citrulline	[20]
HUVEC	100	n.d.	1.9-Fold increase	~50% Increase in L-citrulline	[35]
HUVEC	100	n.d.	23% (Not significant)	n.d.	[54]
BAEC	n.d.	20–430	20% Increase	2-Fold increase of nitrite/nitrate	[39]
HUVEC	100	4000	56% Increase	~50% Increase in L-citrulline	[36]
EA.hy926					

HUVEC: human umbilical vein endothelial cell; n.d.: not determined; PAEC: porcine aortic endothelial cell; BAEC: bovine aorta endothelial cells.

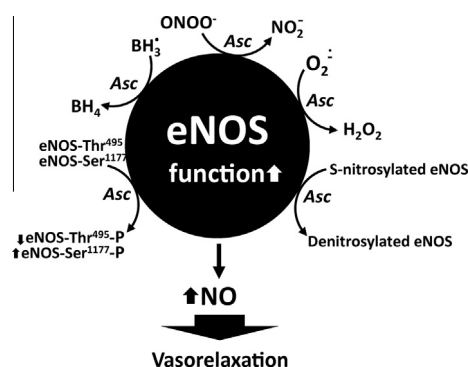


Fig. 3. Mechanisms by which Asc may improve vasorelaxation by increasing NO bioavailability through increased eNOS activity. See text for details.

Asc enhancement of eNOS activity

Based on the *in vitro* and *ex vivo* findings, the effect of Asc on eNOS activity may be summarized as in Fig. 3. Besides the reactions depicted in Fig. 3, Asc might also increase NO bioavailability in an eNOS-independent reaction by reducing nitrite, a degradation product of NO, to NO, a process utilized in the curing of meat. However, this reaction requires a low pH, such as that found in the stomach, to proceed at an appreciable rate [46]. Hence, in the vasculature, enzymatic reduction of nitrite to NO is more likely than Asc-mediated reduction [47].

In vivo studies

Many studies have looked at the effect of either BH₄ or Asc on endothelial function (for recent reviews see [1,48]). These studies have collectively shown that Asc and BH₄ may individually increase the bioavailability of NO *in vivo* and improve clinical endpoints such as blood pressure and blood flow. However, this review will focus on studies in which BH₄ and Asc have been administered and/or assayed together. Such combined experiments have been performed in both humans and animals. Whereas experiments in humans have mainly looked at clinical endpoints, animal experiments have mostly been concerned with measurements of NOS activity.

Animals

Guinea pigs, like humans, lack the ability to synthesize Asc and therefore completely rely on its presence in their diet [49]. Consequently, the guinea pig is a particularly well-suited animal model to study the possible interaction between vitamin C and BH₄. Chatterjee and coworkers placed guinea pigs on a vitamin C deficient diet for two weeks and found that, Asc in plasma and in neutrophils decreased by 80% and 78%, respectively, compared to guinea pigs supplemented with 30 mg/day [37]. As a result of the ascorbic acid-deficient diet, BH₄ levels in neutrophils decreased by 60% [37]. The neutrophils were isolated and used for *ex vivo* studies (see above). Recently, we have demonstrated that plasma biopterin oxidation status is closely linked to the amount of Asc in plasma and hence in the diet *in vivo* [50]. In another study, guinea pigs were fed a diet containing either 100, 250, 500, 750, 1000 or 1500 ppm ascorbic acid. Those receiving 500–1500 ppm Asc had significantly higher plasma levels of BH₄, than those receiving 100 ppm and the group fed 100 ppm Asc also had a higher BH₂-to-BH₄ ratio than all the other groups [50]. A positive linear correlation between plasma BH₄ and plasma Asc was observed, whereas a negative correlation was observed between plasma BH₄ and plas-

ma DHA. BH₂ plasma levels were not correlated with either Asc or DHA, whereas the BH₄-to-BH₂ ratio showed the same correlations as did BH₄ [50].

Commonly used rodents such as mice and rats produce their own vitamin C thereby potentially making supplementation studies difficult to interpret. Regardless, studies have also investigated the relationship between Asc and BH₄ in these species. Similarly to the guinea pigs studies mentioned above, wild-type mice receiving feed containing 1% Asc more than tripled their BH₄ in aorta with no effect on BH₂ levels compared to controls [14]. In ApoE-deficient mice, on the other hand, BH₄ levels in aorta were unaffected by supplementation with Asc, while BH₂ levels decreased; however, neither BH₄ nor BH₂ levels in liver were affected by vitamin C supplementation [14]. In both cases, this resulted in an increased BH₄-to-BH₂ ratio which correlated with increased NOS activity as measured by the formation of L-citrulline [14].

Other studies have looked at the effect of increased bioavailability of Asc and BH₄. Rats received BH₄, L-arginine, BH₄ + L-arginine or BH₄ + L-arginine + ascorbic acid in their diet for one week after which hindlimb ischemia was induced. The rats fed either BH₄ or L-arginine alone showed no improvement of blood flow recovery after induction of ischemia whereas those receiving either BH₄ + L-arginine or BH₄ + L-arginine + Asc showed a significant improvement [44]. This correlated with an increase in collateral artery diameter in rats receiving BH₄ + L-arginine with an even greater increase when Asc was also included in the feed. Nitrite and nitrate, markers of NO, were higher in rats fed BH₄ + L-arginine and BH₄ + L-arginine + Asc, and inclusion of Asc in the diet caused higher levels of nitrite and nitrate though it did not reach statistical significance (compared to the BH₄ + L-arginine group), which correlated with an increase in eNOS expression [44]. Oxidative stress, measured as nitrotyrosine and ratio of glutathione to glutathione disulfide, was also lowest in the group receiving Asc [44]. In another study, ApoE-knockout mice overexpressing eNOS (leading to increased oxidative stress) were crossed with mice overexpressing the rate-limiting enzyme in the biosynthesis of BH₄. This group was compared to ApoE-knockout mice overexpressing eNOS and to ApoE-knockout mice overexpressing eNOS who were given 500 mg ascorbic acid/kg body weight/day [51]. The effect of increased BH₄ biosynthesis was an increase in BH₄ in aorta but without a concomitant increase in BH₄-to-BH₂ ratio; Asc had no effect on BH₄ levels or BH₄-to-BH₂ ratio. BH₄ increased the eNOS dimer-to-monomer ratio and decreased superoxide concentrations – Asc also decreased superoxide concentration but without altering the eNOS dimer-to-monomer ratio [51], indicating that Asc scavenges superoxide rather than changing the propensity for eNOS to produce superoxide. Overexpression of BH₄ led to a decrease in atherosclerotic lesion formation whereas supplementing with Asc had no effect on atherosclerotic lesion formation in ApoE mice [51].

Clinical studies

For years, epidemiological studies have repeatedly shown that a poor vitamin C status is associated with increased risk of developing cardiovascular disease [52]. Fueled by the expanding *in vitro* literature supporting a role of Asc in maintaining BH₄ reduced, we and others have speculated that increased NO availability mediated by reducing equivalents from Asc may be the missing link explaining the clinical observations [52]. However, so far only a very limited number of clinical studies have investigated the relationship between Asc and BH₄.

In one study, people suffering from hypertension (systolic blood pressure >135 mmHg) were given an oral combination of BH₄ and Asc (200 mg of each twice daily or 100 mg of each twice daily for four weeks). A significant decrease in systolic and mean blood pressure and an increase in flow-mediated vasodilation were ob-

served [53]. The effect on blood pressure was ascribed to BH₄ as a two week run-in period with Asc alone did not alter blood pressure. However, a combination effect of BH₄ and Asc could not be ruled out [53].

Mittermayer and co-workers studied the effect of BH₄ and Asc on forearm blood flow (FBF) responses to acetylcholine. Infusion of lipopolysaccharide caused a decrease in FBF response to acetylcholine of 23%, but subsequent infusions of BH₄ (500 µg/min) or Asc (24 µg/min) restored FBF response to baseline values [54]. Asc did not increase systemic levels of BH₄ but did cause a local BH₄ increase in the forearm [54]. In a similar study, the effect of BH₄ and Asc on FBF in chronic smokers was examined. BH₄ infusion (500 µg/min) increased FBF response to acetylcholine and serotonin in smokers but not in non-smokers; Asc infusion (6 or 18 µg/min) also improved FBF response to acetylcholine (the response to serotonin was not examined) [55]. Co-infusion of BH₄ and Asc did not improve FBF compared to either agent alone and infusion of Asc prior to infusion of BH₄ abolished the effect of BH₄ [55].

As reviewed in detail elsewhere [48], vitamin C pharmacokinetics are highly dose dependent and ingestion of about 400 mg/day result in a situation of near-saturation of plasma and tissues [10]. Thus, if adequately supplied through the diet, excess amounts achieved, e.g., by supplementation, are rapidly and effectively excreted. As discussed below, this issue is often overlooked in the clinical literature on vitamin C supplementation [56], and notably, none of the studies mentioned above enrolled subjects that were vitamin C deficient at study start. Consequently, they give no information on whether poor vitamin C status—a situation affecting substantial proportions of the World's population including the industrialized parts [56]—negatively affects the bioavailability of BH₄ or if poor BH₄ status can be ameliorated by vitamin C supplementation.

Looking more generally at cardiovascular health, almost all of the large phase III randomized controlled trials using vitamin C as intervention have not been able to demonstrate effect of vitamin C supplementation on cardiovascular morbidity or mortality [57–61]. This constitutes a considerable discrepancy to the experimental and epidemiological literature. However, while experimental and epidemiological literature is generally weakened by their insufficient predictive validity, lack of ability to demonstrate causality in humans, and risk of unidentified confounders and co-deficiencies, the majority of the large clinical intervention studies suffers from several serious design issues including e.g., absence of vitamin C deficiency as inclusion criterion, no record of vitamin C status before or after intervention, and unrecorded concurrent supplementation with vitamin C – also among placebo allocated individuals [52]. Thus, based on the information provided, the major vitamin C intervention studies with cardiovascular endpoints have the serious weakness of studying populations with adequate to high vitamin C levels at baseline. This renders them only able to confirm that supplementation to already well-nourished individuals is very likely to be without effect. Unfortunately, no trials have so far systematically studied the effect of vitamin C deficiency on cardiovascular risk or the effect of supplementation in this particularly relevant subpopulation. Consequently, it is not presently possible to evaluate to what extent the potential effects of vitamin C on BH₄ status found experimentally are clinically relevant.

Conclusion

Asc has been shown to improve vasorelaxation in humans and animals. A number of studies have indicated that rather than a single mechanism, several different mechanisms appear to be involved in Asc-mediated vasorelaxation. Mainly, Asc may improve endothelial function by increasing NO bioavailability through BH₄

and eNOS. Asc may increase BH₄ bioavailability by protecting BH₄ from being oxidized by scavenging ROS and/or by reducing oxidized BH₄. Asc may also increase eNOS activity by changing its phosphorylation and S-nitrosylation status. However, although shown to occur *ex vivo*, it remains to be established to which extent these mechanisms are important *in vivo*.

Conflicts of interest

The authors declare no conflict of interest that could influence the present work.

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