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Review Article

Ascorbate and plasma membrane electron transport—Enzymes vs efflux

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ABSTRACT

Transplasma membrane electron transport (tPMET) systems transfer electrons across the plasma membrane, resulting in the net reduction of extracellular oxidants (e.g., ferricyanide) at the expense of intracellular reductants such as NADH and ascorbate. In mammalian tPMET systems, the major proximal electron donor is ascorbate. The classical description of ascorbate-dependent tPMET views ascorbate as a restrictively intracellular electron donor to a transplasma membrane enzymatic activity that transfers electrons across the plasma membrane to various physiological acceptors (e.g., ferric iron and the ascorbyl radical). Candidate proteins involved in this process include members of the cytochrome b_{561} family (e.g., duodenal cytochrome *b*). However, mounting evidence suggests that cellular export of ascorbate (and concomitant import of its two-electron oxidation product, dehydroascorbate) may constitute a novel and physiologically relevant form of ascorbate-dependent tPMET. As with enzymatic tPMET, cellular ascorbate export results in net electron transfer from the cytoplasm to the extracellular space. The mechanisms of ascorbate release from cells are ill-defined, though volume-sensitive anion channels and exocytosis remain promising candidates. Cellular ascorbate release is implicated in various homeostatic processes including ascorbate maintenance in blood and brain, and the uptake of non-transferrin-bound iron by cells. Recent insights into the "duality" of ascorbate-dependent tPMET are discussed.

Contents

Abbreviations: AFR, ascorbyl free radical; AO, ascorbate oxidase; Dcytb, duodenal cytochrome *b*; DHA, dehydroascorbate; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; DDF, dideoxyforskolin; EAAT, excitatory amino acid transporter; GLO, gulono-γ-lactone oxidase; GLUT, facilitative glucose transporter; Lcytb, lysosomal cytochrome *b*; NPPB, 5-nitro-2-(3-phenylpropylamino) benzoic acid; SDR2, stromal cell-derived receptor 2; SITS, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid; tPMET, transplasma membrane electron transport; SVCT, sodium-ascorbate cotransporter; VSOAC, volume-sensitive osmolyte and anion channel.

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Introduction

Transmembrane electron transport in eukaryotes was once typically associated with respiratory and photosynthetic electron transport in the internal membranes of mitochondria and chloroplasts, respectively. However, the existence of electron transfer reactions in other cellular membranes-in particular the plasma membrane—is now well documented [1–6]. The concept of transplasma membrane electron transport (tPMET) is suggested by the canonical observation that plasma membrane-impermeant oxidants such as ferricyanide can be reduced by metabolically active cells possessing an intact cell membrane. These activities have since been related to the regulation of vital cellular processes including growth control and differentiation [1,2], pH control and mitogenesis [2,4], bioenergetics [7,8], cell signal transduction [2], apoptosis [9,10], antioxidation [3,11], and iron/copper metabolism [1,2,4,12,13]. Consistent with these roles, deregulation of tPMET may contribute to the pathogenesis of various human conditions including aging and neurodegeneration [14,15], macrophage-mediated LDL oxidation in atherogenesis [16], diabetic nephropathy [17], and glycolytic cancer progression [18–20]. The most researched intracellular electron donors for mammalian tPMET are ascorbate [3,6] and NADH [1,4,21]. Historically, much of the early research focused on the NADH-dependent system(s) [1,2,4,21]. Despite this, it is now clear that though NADH is certainly involved in electron donation in mammalian tPMET, and may further be involved in the regeneration of intracellular ascorbate, the majority of electrons probably derive proximally from ascorbate in many cell types [3,6,22–24].

This review focuses on recent data and insights into the mechanisms and functions of ascorbate-dependent tPMET. In particular, we examine the notion that ascorbate-dependent tPMET occurs at two mechanistically distinct levels: (i) enzyme-mediated transmembrane electron transfer and (ii) transplasma membrane ascorbate efflux. Both of these mechanisms essentially function to transport reducing equivalents derived from cellular metabolism via ascorbate to the extracellular space for deployment in a range of physiological functions ranging from iron uptake to antioxidative defense. Recent insights into the mechanisms and physiological relevance of each system are discussed.

Ascorbate: Redox reactions and cellular uptake

Ascorbate redox in mammals—A brief overview

As a physiological reductant and enzyme cofactor, L-ascorbate contributes to numerous well-defined enzymatic reactions involving collagen hydroxylation, carnitine and norepinephrine biosynthesis, tyrosine metabolism, and peptide hormone amidation [25]. Ascorbate also contributes significantly to cellular antioxidation as a water-soluble chain-breaking radical scavenger [26] and to the recycling of plasma membrane α-tocopherol (vitamin E) via the reduction of the α-tocopheroxyl radical [27]. The latter activity may assist ascorbate to protect against lipid peroxidation in membranes [28]. Most mammals are capable of de novo hepatic synthesis of ascorbate from glucose, through a biosynthetic pathway that employs the enzyme gulono-γ-lactone oxidase (GLO) for the terminal oxidation reaction [29–32]. However, higher primates, guinea pigs, and some bats are obligatorily dependent on dietary sources of the vitamin [33], due to inactivation of the gene encoding GLO [30,31,34]. Following absorption from the intestinal lumen in humans, ascorbate traverses the body in the circulatory system and is typically found at millimolar concentrations intracellularly (nucleated cells), and at micromolar concentrations in extracellular fluids and erythrocytes [35,36].

Under physiological conditions, ascorbate typically undergoes a reversible one-electron oxidation to the ascorbyl free radical (AFR; also known as monodehydroascorbate or semidehydroascorbate). The AFR is chemically stable as far as radicals go [37] and is also enzymatically recyclable (discussed in depth below). However, in the absence of enzymatic reduction of AFR to ascorbate, two AFRs can further dismutate to one ascorbate and one DHA [3,32,34] (Fig. 1). If utilized, AFR dismutation effectively results in each ascorbate molecule being able to donate two reducing equivalents in cellular redox reactions. Within cells DHA is rapidly reduced back to ascorbate by GSH- and NAD(P)H-dependent enzymatic and nonenzymatic reactions [32]. The

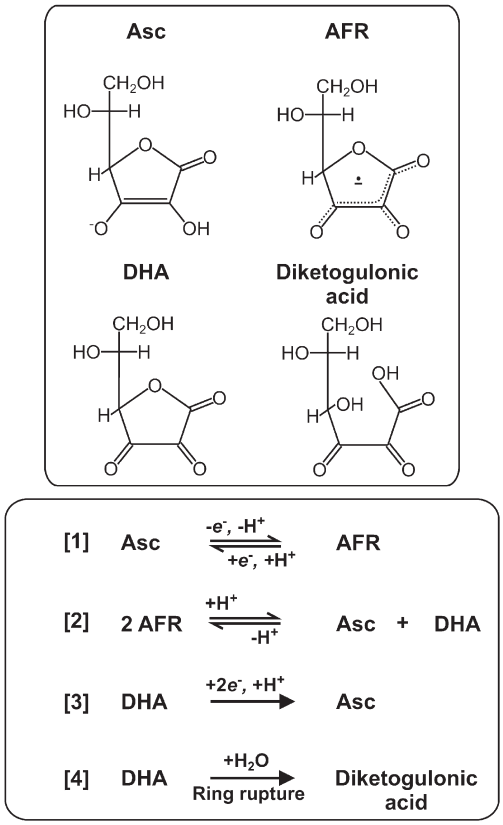

Fig. 1. The major biological redox reactions of ascorbate and its oxidation products. The ascorbate monoanion (Asc) is the predominant species of vitamin C present under physiological conditions. Asc can undergo a thermodynamically favorable and reversible one-electron oxidation to the ascorbyl free radical (AFR; Reaction 1). AFR is stabilized by resonant distribution of the resultant unpaired electron over the ring structure and can be enzymatically recycled back to Asc. If not rapidly reduced, two AFRs can dismutate to form one molecule each of the two-electron oxidized form, dehydroascorbate (DHA; Reaction 2) and Asc. DHA can be reduced within the cell interior back to Asc in a two-electron reduction, which bypasses AFR (Reaction 3). DHA is highly unstable, and if not rapidly reduced back to Asc, undergoes an essentially irreversible hydrolytic ring opening to 2,3-diketogulonic acid with a half-life of several minutes under physiological conditions (Reaction 4).

mitochondrial electron transport chain is also an important site of intracellular DHA reduction in nucleated cells [38–43], with reduction taking place at an apically exposed section of complex III in mammals [40]. DHA reduction appears to occur as a two-electron reduction step that bypasses the intermediate production of AFR [3]. Intracellular ascorbate regeneration from either AFR or DHA has been reviewed recently by Linster and Van Schaftingen [32].

The inherent disadvantage with the complete oxidation of ascorbate to DHA is that DHA is structurally unstable, with a half-life of several minutes under physiological conditions [44–46]. The rapid degradation by ring opening of DHA to diketogulonic acid results in an irrevocable loss of the vitamin from mammalian systems [44–46]—a point that is particularly pertinent to species lacking GLO activity. In order to cope with this degradative tendency, vitamin C is maintained predominantly in its fully reduced form in both intra- and extracellular biological fluids. As referred to above, mammalian cells possess a variety of conservative reductive mechanisms for maintaining both intra- and extracellular ascorbate [3,32,34,47,48]. Even cultured cells—which are usually chronically ascorbate-deficient due to lack of supplementation and/or biosynthesis under standard culture conditions [49,50]—still maintain an extraordinary ability for ascorbate regeneration from both AFR and DHA [32]. The net effect of this regeneration is the maintenance of physiological ascorbate concentrations within biological fluids (e.g., plasma, interstitial, and cerebrospinal fluid) at the expense of other, predominantly intracellular, reductants (e.g., GSH and NAD(P)H).

Ascorbate uptake by cells—SVCTs vs GLUTs

The major routes of ascorbate uptake in mammalian cells have been reviewed previously [35,51], but will be summarized here to aid the clarity of the ensuing discussion. The majority of mammalian cells—with the notable exception of human erythrocytes [36]—maintain intracellular ascorbate concentrations that are markedly higher [e.g., up to 30-fold in some cases] than those in the extracellular fluid [3,34,35]. For instance, lymphocytes accumulate intracellular ascorbate to concentrations of approximately 4 mM in the face of plasma concentrations of 40–80 μM [52], while neurons achieve intracellular ascorbate concentrations of up to 10 mM in the face of extracellular concentrations of 200–400 μM [53,54]. This outward-facing concentration gradient is generated predominantly by sodium-dependent import of ascorbate into cells by sodium-ascorbate cotransporters (SVCTs 1 and 2) [51] that utilize the sodium concentration differential across the plasma membrane (Fig. 2A). Human SVCT1 (K_M range: 65–237 μM) is expressed mostly in apical membranes of intestinal epithelial cells and renal tubular cells (assisting in absorption and reabsorption of the vitamin, respectively), while human SVCT2 (K_M range: 8–62 μM) is expressed in most other nucleated cells of the body [51]. SVCT2 expression within the brain is vital for brain function and ascorbate homeostasis in this organ [55]. The result of near ubiquitous SVCT2 expression throughout the body is that ascorbate transport by this transporter is close to saturation at the ascorbate levels found in plasma and extracellular fluid. Cells can also accumulate intracellular ascorbate against a concentration gradient via lower affinity, higher capacity transport of DHA through facilitative glucose transporter isoforms 1, 3, and 4 (GLUTs 1, 3 and 4) [35,56–59] (Fig. 2A). With respect to DHA uptake by cells, an inward-facing DHA gradient is maintained by the rapid reduction of imported DHA back to ascorbate, the latter of which is a poor substrate for GLUT-mediated transport [23,35,60].

An adaptation for DHA import in human erythrocytes—GLUT1 and stomatin

Unlike nucleated cells, human erythrocytes maintain intracellular ascorbate concentrations equivalent to that in plasma [61]. This appears to be due to the loss of ascorbate transport that coincides with loss of SVCT2 expression during development of the mature erythrocyte [36]. Erythrocytes can, however, efficiently accumulate intracellular ascorbate from extracellular DHA [23,61]. Moreover, typical circulating DHA levels are relatively low (<2 μM) [34] and human erythrocytes apparently must achieve their observed intracellular ascorbate concentrations by DHA import and subsequent reduction to ascorbate.

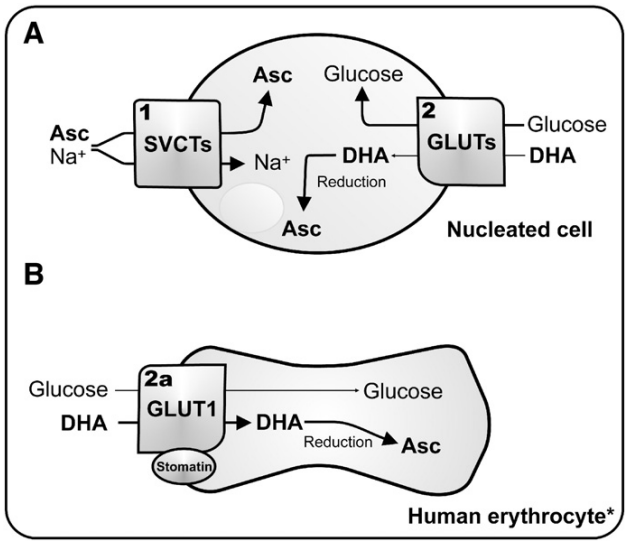

Fig. 2. Routes of vitamin C import by mammalian cells. (A) Two main pathways exist for vitamin C import into mammalian cells. The ascorbate (Asc) pathway (1) imports ascorbate in a sodium-dependent manner via SVCTs (i.e., SVCTs 1 and 2) in many nucleated cells. The DHA pathway (2) imports DHA via GLUTs (i.e., GLUTs 1, 3, and 4) in a sodium-independent manner. (B) In human erythrocytes the association of the integral plasma membrane protein stomatin (band 7.2b) with GLUT1 switches the import preference from D-glucose to L-DHA uptake, resulting in DHA transport that is not competitively inhibited by physiological glucose concentrations (2b). In both cases (i.e., 2 and 2a), imported DHA is rapidly reduced to Asc in the cell interior by a variety of enzymatic and nonenzymatic reductive mechanisms. *The association between GLUT1 and stomatin appears to be found only in erythrocytes from species that are unable to synthesize their own ascorbate (e.g., simians, including humans, and guinea pigs), and thus appears to be an evolutionary adaptation to the lack of endogenous ascorbate production [62].

An intriguing recent report [62] suggests that, unlike most nucleated cells and erythrocytes from species capable of vitamin C biosynthesis, erythrocytes from ascorbate auxotrophs (e.g., humans) are biochemically "adapted" to allow DHA transport that is unimpeded by physiological glucose concentrations. This adaptation arises from the association of GLUT1 with the integral membrane protein, stomatin (band 7.2b) [62] (Fig. 2B), and appears to be a compensatory regenerative mechanism for the lack of endogenous ascorbate production [62–66]. This mechanism explains the seemingly paradoxical observations that DHA import by human erythrocytes is sensitive to pharmacological GLUT1 inhibition [47], yet is glucose-insensitive [23]. It was already known that stomatin coimmunoprecipitates with GLUT1 in human erythrocyte membrane extracts [67] and that this association is mediated by a protein–protein interaction between stomatin and a C-terminal peptide of GLUT1, which greatly inhibits intrinsic glucose transport activity by up to 50% [68]. The significance of these observations is not yet clear, though it is likely to be related to the lack of endogenous ascorbate production in GLO-deficient species [62,63]. That is, erythrocytes from such species—by virtue of the association of GLUT1 with stomatin—may be better able to assist in the regeneration of ascorbate from its oxidized forms than erythrocytes from ascorbate producing species in which these cells are not optimized for uninhibited DHA import. For example, erythrocytes from GLO-deficient species such as humans may perform a crucial ascorbate recycling function in plasma and tissues. This process may

be particularly pertinent to tissues experiencing heightened levels of oxidative stress (e.g., at sites of vascular inflammation [27,36]). The capacity of human erythrocytes to regenerate extracellular ascorbate under normal and oxidative stress conditions will be discussed later in this review in the context of both enzymatic and efflux-based ascorbate-dependent tPMET.

Ascorbate and enzymatic tPMET

Much of the research carried out to date on ascorbate-dependent tPMET has proposed an enzymatic electron transfer mechanism across the plasma membrane (for reviews, see May [3] and Kennett and Kuchel [6]). Though ferricyanide has frequently been used as an analytical tool to measure ascorbate-dependent tPMET, it is clearly a nonphysiological substrate. Several physiological electron donors have been proposed including extracellular AFR and low molecular weight chelates of iron and copper (see below). Though the proteins involved in mediating these activities are yet to be conclusively identified, there is mounting evidence that recently described members of the cytochrome b_{561} family—in particular duodenal cytochrome b (Dcytb)—are involved [11–13,69–71]. We now discuss recent insights into the mechanisms and functions of enzymatic ascorbate-dependent tPMET with a focus on past seminal functional studies and on the emerging view that Dcytb may be an important mediator of this activity.

Ascorbate and cellular ferricyanide reduction—Establishing a general mechanism

The trivalent ferricyanide anion is commonly used as an artificial electron acceptor for the assay of tPMET, due to its physiological redox potential ($E_0' = 360$ mV—a redox potential similar to both AFR and low molecular weight ferric chelates), excellent aqueous solubility, and lipid bilayer impermeance [3,72], as well as the ease and sensitivity of determination of its reduced form, ferrocyanide [24,73]. Increasing intracellular ascorbate concentration stimulates extracellular ferricyanide reduction by a variety of primary and secondary cell types, including human erythrocytes [3,6,22,47,74], K562 cells [24,75], HL60 cells [76], U937 cells [77], and pulmonary arterial endothelial cells [78]. The mechanism of this stimulation, however, has long been a contentious issue. The most prominent proposals include (i) the existence of extracellular ferricyanide-supported redox cycling of ascorbate at the cell surface by either AFR-reductase [75] and/or DHA-reductase [23,79] activities (Fig. 3A), (ii) the release of intracellular ascorbate into the extracellular space followed by direct chemical reduction of ferricyanide [80] (Fig. 3B), and (iii) a transmembrane ascorbate-dependent ferricyanide reductase activity that utilizes intracellular ascorbate for electrons [3,6,24,77] (Fig. 3C).

Proposal 1 is unlikely for the following reasons. First, though ferricyanide is easily reduced directly by ascorbate, AFR concentrations are virtually undetectable in the presence of analytical ferricyanide concentrations [76,81]. This suggests that the oxidizing power of excess ferricyanide drives the redox equilibrium almost entirely to DHA. Second, though an AFR-reductase activity of plasma membranes is well documented [6,11,69,82–84], the lack of statistically significant inhibition of ferricyanide reduction by the enzyme ascorbate oxidase (AO) [77,78]—which facilitates complete oxidation of ascorbate to DHA—further argues against this possibility. Furthermore, the contribution of an exofacial plasma membrane DHA reductase activity [79] has only indirect experimental support. Addition of DHA to K562 cells [79] and human erythrocytes [23] results in the appearance of extracellular ascorbate. This observation has been taken to imply that DHA can be reduced extracellularly by cells [23,79]. If cells can reduce DHA extracellularly to ascorbate, the ascorbate formed could potentially react directly with added ferricyanide, thus giving the appearance of a transmembrane ascorbate:ferricyanide oxidoreductase activity. Against this interpretation, however, we have recently shown that—at least in the case of K562 cells—the appearance of extracellular ascorbate in response to added DHA is entirely dependent on DHA uptake and at least partially dependent on ascorbate release from the intracellular compartment [85]. Similar results were previously obtained with both U937 [77] and HepG2 [86] cells. These observations argue against the universality of the mechanism suggested by proposal 1 and suggest instead that alternative mechanisms may be required to explain the original experimental observations.

Proposal 2 was put forward as an early explanation of DHA-stimulated ferricyanide reduction in human erythrocytes [80]. Orringer and Roer [80] originally observed a strong positive correlation between intracellular ascorbate concentration and the rate of ferricyanide reduction by metabolically active human erythrocytes. As extracellular DHA accumulation accompanies ferricyanide reduction by these cells [23,47,80] it was reasoned that ferricyanide reduction was mediated by ascorbate export from cells followed by the direct chemical reduction of two molecules of ferricyanide per molecule of ascorbate [80]. May and colleagues [22,47] later provided for a plausible reinterpretation of these data by suggesting that reduction of extracellular ferricyanide by ascorbate-loaded erythrocytes was

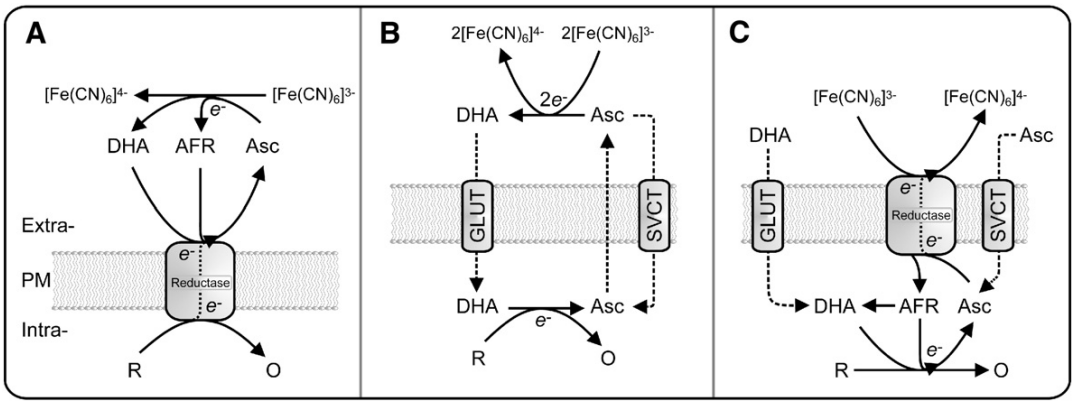

Fig. 3. Alternative models for ascorbate-mediated stimulation of cellular ferricyanide reduction in mammals. (A) Ascorbate (Asc) may stimulate extracellular ferricyanide ($[Fe(CN)_6]^{3-}$) reduction to ferrocyanide ($[Fe(CN)_6]^{4-}$) via enzymatic, tPMET-supported redox cycling of extracellular Asc species (i.e., Asc, DHA, and AFR) at the plasma membrane (PM). (B) Alternatively, intracellular Asc (resulting from either GLUT-mediated DHA import and subsequent reduction or SVCT-mediated Asc import) released from cells may quantitatively reduce extracellular ferricyanide to ferrocyanide. (C) The most prominent mechanism of Asc-stimulated extracellular ferricyanide reduction appears to result from intracellular Asc oxidation coupled to electron flow through the redox centers of a transplasma membrane ferricyanide reductase. In all panels, 'R' and 'O' together refer to an unspecified, not necessarily identical, cytoplasmic redox couple. Please note that all three models are not mutually exclusive.

caused rather by intracellular oxidation of ascorbate and subsequent GLUT-dependent efflux of DHA. This interpretation was largely reaffirmed in ^{13}C NMR-based studies of the ascorbate-stimulated ferricyanide reduction reaction by metabolically intact human erythrocytes [6,87]. Moreover, the recurrent observations that cell-conditioned medium of ascorbate-supplemented cells fails to elicit stimulation of ferricyanide reduction [3,24], and that treatment with AO is not inhibitory [77,78], further argue against the "transport" model as a general mechanism for ascorbate-stimulated ferricyanide reduction by cells. Instead, these data collectively support the hypothesis that ascorbate-dependent reduction of extracellular ferricyanide relies primarily on an enzyme-based tPMET pathway from intracellular ascorbate to extracellular ferricyanide, as previously proposed for human erythrocytes [3,6,22,87,88]. As discussed in the next section, the basic enzymatic model outlined in proposal 3 (Fig. 3C; see below) is further supported by the identification of the putative ascorbate-dependent oxidoreductase, Dcytb.

Ascorbate and cytochromes b_{561}—An emerging role for Dcytb

The identities of the enzymes responsible for ascorbate-dependent electron transfer across the cell membrane to extracellular oxidants such as ferricyanide and AFR remain unknown. However, related studies on ferrireductases and iron uptake have suggested a likely, but as yet undemonstrated, candidate. The discovery of Dcytb in 2001 by McKie and colleagues [12] as a putative ferrireductase in duodenal enterocytes paved the way for a number of studies that implicate this protein in ascorbate-dependent transmembrane electron transfer. Dcytb belongs to the widespread cytochrome b_{561} family, which has members in all eukaryotic kingdoms [69,89]. These proteins constitute an ever growing family of di-heme *b*-containing transmembrane redox proteins that bear both structural and functional homology to the classical cytochrome b_{561} of chromaffin granule membranes [12,69,89]. Cytochrome b_{561} is most well known for its role in the transmembraneous electron transfer from cytosolic ascorbate to intravesicular AFR in neuroendocrine secretory granules [90]. The intravesicular ascorbate that results from this electron transport is subsequently reoxidized to AFR in the intravesicular redox reactions catalyzed by copper-containing dopamine β-hydroxylase and peptidyl glycine α-amidating monooxygenase [89]. The reaction catalyzed by cytochrome b_{561} is thought to involve a histidine cycling mechanism of coupled proton/electron transfer between ascorbate and AFR [91]. Additional members of this family of redox enzymes in mammals include lysosomal cytochrome b_{561} (Lcytb) [69,92], stromal cell-derived receptor 2 (SDR2) [93], and gene product 101F6 [94]. The remainder of this section of the review will focus on Dcytb, as it is demonstrably present in the plasma membrane [11,12] and appears to be the most likely candidate for an ascorbate-dependent transplasma membrane oxidoreductase.

Is Dcytb an ascorbate-dependent ferrireductase?

Though originally discovered in the brush-border membrane of duodenal enterocytes [12], Dcytb now appears to be widely expressed in a variety of tissue and cell types including human erythrocytes [11], lung epithelial cells [70], K562 cells [95,96], and Hep-G2 and Caco-2 cells [96], as well as astrocytes [97]. This suggests that the functions served by Dcytb may be of a more general importance to cell biology. In support of this notion, a growing number of studies [12,13,70,71,98–100] have implicated Dcytb as a ferrireductase in the reduction of non-transferrin-bound iron prior to ferrous-selective iron uptake by cells. Importantly, as Dcytb demonstrates partial conservation of the canonical ascorbate binding motif originally identified in cytochrome b_{561} [69,89], ascorbate is a likely proximal electron donor for this activity. Accordingly, several studies [13,69,71,98] in which Dcytb expressing cells have been supplemented with ascorbate or DHA have provided experimental support for this conclusion. The model espoused as a result of these studies suggests that Dcytb can catalyze electron transfer from intracellular ascorbate directly to extracellular ferric or cupric ions [12,13,69,71,101] (Fig. 4A).

Importantly, these studies have *not* excluded the possibility that extracellular ascorbate is involved in the mechanism of ferrireduction. For example, though extracellular ascorbate is not required for the stimulation of ascorbate-dependent ferricyanide reduction by human erythrocytes, extracellular ascorbate *does* appear to be required for the ascorbate-dependent stimulation of non-transferrin-bound iron reduction by at least some cell types [77,85]. Therefore, due to the release of significant levels of ascorbate from ascorbate-loaded cells during exposure to non-transferrin-bound iron [77,85], control studies in which AO is added to remove the effects of extracellular ascorbate [13] should ensure that the enzyme is present *during* ferrireduction assays to properly exclude involvement of effluxed ascorbate.

As discussed in the next section, the rising possibility that Dcytb may function as an ascorbate-dependent AFR reductase also needs to be considered in future studies of Dcytb's involvement in ferrireduction.

Is Dcytb an ascorbate-dependent AFR reductase?

Dcytb also shows partial conservation of a putative AFR binding site, suggesting that, as with cytochrome b_{561}, AFR is a likely native electron acceptor. When mapped onto the currently accepted six

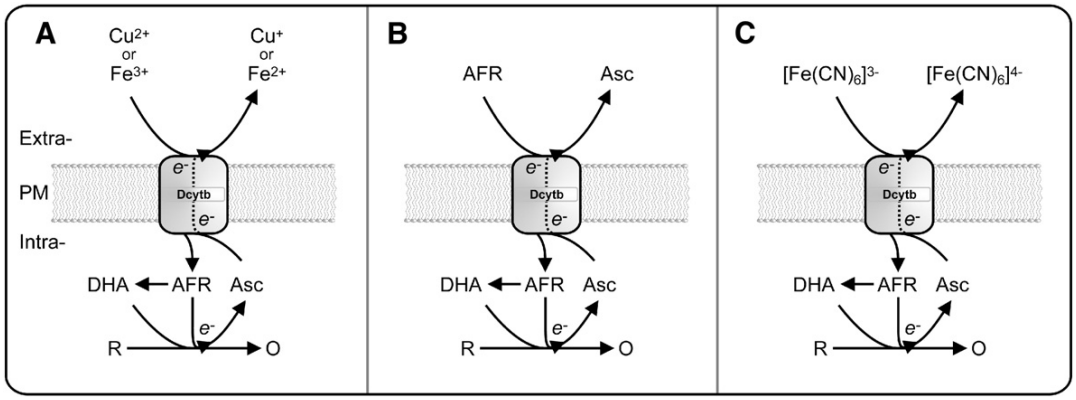

Fig. 4. Possible redox reactions carried out by Dcytb. (A) Dcytb may function as ferric (Fe^{3+}) and/or cupric (Cu^{2+}) reductase, by which ferric and cupric ions are reduced monoelectronically to ferrous (Fe^{2+}) and cuprous (Cu^{+}) ions, respectively, at the cost of intracellular reducing equivalents derived proximally from ascorbate. (B) Dcytb may also function as an AFR reductase, helping to recycle extracellular ascorbate. (C) Dcytb may additionally be the long sought identity of the transplasma ferricyanide reductase of mammalian cells. Please note that all three possible functions are not mutually exclusive.

transmembrane topology model [69,89] for cytochrome b_{561}, the putative ascorbate binding site in Dcytb maps to the cytosolic side of the membrane while that of the AFR binding site maps to the extracellular face [69,89]. This indicates that the corresponding binding sites for ascorbate and AFR are at least oriented with respect to the plasma membrane in a manner consistent with catalysis of ascorbate:AFR oxidoreduction. Though this hypothesis has yet to be directly tested with reconstituted enzyme or heterologous expression systems, Dcytb has recently been suggested by Su and colleagues [11] to function as ascorbate-dependent AFR reductase in human erythrocytes. Intriguingly, these authors further observed that Dcytb is also present in erythrocytes from guinea pigs—which cannot produce their own ascorbate—but not erythrocytes from ascorbate-producing rats and mice. Additionally, Dcytb-containing human erythrocytes were much more effective at preserving extracellular ascorbate than mouse erythrocytes. It is thus tempting to suggest—in line with the above discussions on the putative ascorbate-conserving adaptations of erythrocytes from GLO-deficient species—that the presence of Dcytb in erythrocytes from such species is a further adaptation toward ascorbate conservation in the face of an inability to biosynthesize the vitamin.

If Dcytb can be demonstrated to function as a transplasma membrane ascorbate:AFR oxidoreductase in cells, the observed ability of Dcytb to stimulate ascorbate-dependent reduction of iron and copper may utilize a subtly different mechanism to that previously proposed. That is, in the presence of catalytic concentrations of ascorbate, Dcytb may catalyze electron transfer from intracellular ascorbate to extracellular AFR to generate ascorbate, the latter of which could then directly donate a single electron to a ferric or cupric ion (Fig. 4B). This type of ascorbate cycling mechanism is consistent with the presence of both ascorbate and AFR binding motifs in the Dcytb sequence, and is analogous to the above discussed mechanism of action of cytochrome b_{561} in neuroendocrine secretory vesicles. Though both models propose a dependence on intracellular ascorbate, the novel model outlined here includes an additional dependence on a catalytic pool of extracellular ascorbate. This model could easily be tested by judicious use of AO in ferrireductase assays involving ascorbate-loaded cells (e.g., Lane and Lawen [85]).

Is Dcytb a transplasma membrane ferricyanide reductase?

Heterologously expressed Dcytb facilitates ascorbate-dependent ferricyanide reduction [69]. Su and Asard [69] were able to show that yeast heterologously expressing Dcytb shows greatly enhanced rates of ferricyanide reduction, but only when supplemented with a biosynthetic precursor of ascorbate. These authors also assessed a range of point mutations affecting the histidines involved in heme coordination and in the ascorbate binding site in the sequence of Lcytb—a Dcytb homologue demonstrating similar ascorbate-dependent ferricyanide reductase to Dcytb. Many of these mutations were able to markedly inhibit ferricyanide reduction [69]. Interestingly, point mutations in the putative AFR binding site affected ferricyanide reduction to a much lesser degree, suggesting that ferricyanide may be able to accept electrons directly from the exofacial heme by bypassing the AFR binding site. Again comparative studies [11] between human erythrocytes (which contain Dcytb) and mouse erythrocytes (which lack Dcytb) demonstrate that the former have much higher rates of ferricyanide reduction. Collectively these studies suggest that Dcytb is at least capable of functioning as a transplasma membrane ascorbate:ferricyanide oxidoreductase (Fig. 4C), and that Dcytb is a good candidate for this activity in human erythrocytes. However, further experiments (e.g., RNA interference studies) are needed to establish the extent to which this activity is attributable to Dcytb in other cell types. The utility of establishing Dcytb's involvement in enzymatic ascorbate-dependent tPMET will be that, given the relative difficulty of analyses involving AFR, ferricyanide could potentially be used as an analytical tool for further functional and kinetics studies of the enzyme's redox activity.

Ascorbate and efflux-dependent tPMET

Despite the historical focus on enzymatic ascorbate-dependent tPMET, a growing number of reports document the regulated release of ascorbate from the intracellular compartment to the extracellular space [35,85,86,102–104]. As intracellular ascorbate is recycled at the expense of reducing equivalents derived from cellular metabolism, ascorbate release constitutes an additional form of ascorbate-dependent tPMET. As with the enzyme-mediated tPMET systems described above, the net effect of ascorbate efflux on cellular redox is the export of metabolically derived reducing equivalents from the cytoplasmic compartment for deployment in the extracellular space [58,85,86,102,103,105,106]. Once in the extracellular space, however, the fate of these reducing equivalents is probably multitudinous and will depend on the cellular context. Unfortunately, relatively little is known about the mechanisms of this release. Recent insights into the mechanisms and possible physiological functions of ascorbate release by cells are reviewed in this section.

Ascorbate export—Plasma membrane conduits or exocytosis?

The simple diffusion of ascorbate (or DHA) across the lipid bilayer of the plasma membrane is thermodynamically improbable, due to the size and negative charge of the ascorbate monoanion (and the unfavorable oil:water distribution coefficient of DHA) [35]. As with ascorbate import, the routes of ascorbate export appear to be homeostatically regulated. Despite relatively little dedicated research on the mechanisms involved, several plausible candidates for a release pathway have arisen (reviewed previously by Wilson [35]). These include (i) volume-sensitive anion channels [35,104,106,107], (ii) ascorbate–ascorbate homeoexchange [108,109], (iii) gap junction hemichannels [110,111], (iv) glutamate–ascorbate heteroexchange [112], and (v) exocytosis of ascorbate-containing vesicles [113] (Fig. 5).

Volume-sensitive anion channels

The release mechanism that has received the most experimental support over the past decade is the "volume-sensitive anion channel" hypothesis (previously reviewed by Wilson [35]). Volume-sensitive osmolyte and anion channels (VSOACs) are functionally defined, protein-dependent plasma membrane anion channels that are involved in regulatory volume decrease of cells following, for example, cellular swelling caused by hypotonic shock [114]. Electrophysiological evidence indicates that VSOACs are indeed permeable to ascorbate [114] (1 in Fig. 5), though the molecular identities of these channels remain uncertain [115].

Anion channel blocker-sensitive ascorbate efflux has been observed in a variety of cell types, including astrocytes [107,116], hepatocyte-like HepG2 cells [86], SH-SY5Y neuroblastoma cells [104], coronary artery endothelial cells [102,103], and K562 cells [85]. Thus, the frequent observation that both VSOAC permeability [117] and ascorbate efflux from cells can be inhibited by the application of generic anion channel inhibitors, such as 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) and 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS) [85,86,104,107], and the VSOAC-inhibitors dideoxforskolin (DDF) and 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB), suggests that a significant proportion of ascorbate release may occur via this pathway. Moreover, the hypotonic stimulation of ascorbate release from astrocytes [116] and coronary artery endothelial cells [103] by cell swelling further supports this hypothesis. The lack of knowledge on the proteins involved in VSOAC formation and the consequent lack of specific inhibitors of VSOAC activity are important drawbacks in the assessment of VSOAC involvement in ascorbate release. Furthermore, as putative VSOAC involvement accounts for less than 50% of the ascorbate released by many of the cells tested, including hepatocyte-like Hep-G2 cells [86], SH-SY5Y neuroblastoma cells

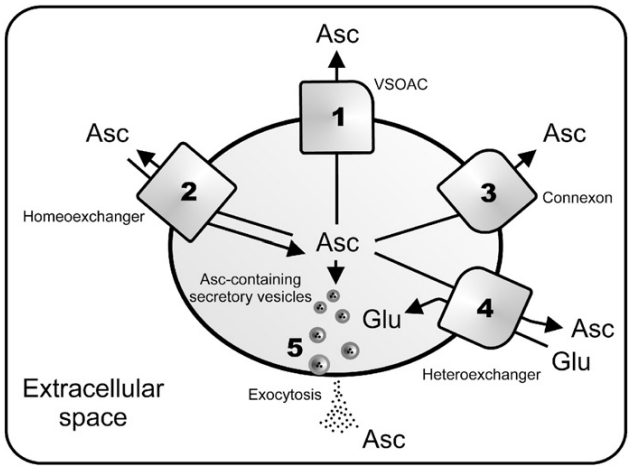

Fig. 5. Alternative possible routes of ascorbate release from mammalian cells. Several plausible candidates for an ascorbate (Asc) release pathway have been proposed, including (1) volume-sensitive osmolyte and anion channels (VSOACs), (2) ascorbate–ascorbate homeoexchange, (3) gap junction hemichannels composed of connexin hexamers (connexons), (4) glutamate–ascorbate heteroexchange, and (5) regulated exocytosis of Asc-containing vesicles. As these alternative release routes are not mutually exclusive, one or more may be operational in any given cell type.

[104], and K562 cells [85], other release pathways are probably significant contributors (see below).

Ascorbate–ascorbate homeoexchange

The observation that addition of extracellular ascorbate to cells promotes release of preloaded [^{14}C]ascorbate has suggested the existence of a cell surface ascorbate–ascorbate homeoexchange mechanism (2 in Fig. 5) in adrenal cortical cells [108] and retinal pigment cells [109]. However, no transporters have been identified that might mediate such an activity. In the above studies, the release of radioactive probe was not dependent on, but was greatly stimulated (up to 10-fold in adrenal cortical cells) [108] by the presence of extracellular ascorbate. May and Qu [118] recently also observed a similar dose-dependent stimulation of efflux of radiolabeled ascorbate by unlabeled extracellular ascorbate from EA.hy926 endothelial cells. This apparent enhanced efflux of ascorbate in response to extracellular ascorbate (which has been taken as evidence for ascorbate–ascorbate homeoexchange) may simply be an artifact of differences in SVCT-dependent re-uptake of effluxed radiolabeled ascorbate in the presence of an excess of unlabeled ascorbate [118]. That is, re-uptake of effluxed radiolabeled ascorbate by SVCTs would be expected to be lower in the presence of excess unlabeled ascorbate (due to dilution effects), giving the appearance of enhanced ascorbate efflux. This issue could be further resolved with pharmacological (e.g., sulfinpyrazone) and/or RNA interference-based inhibition of SVCT-dependent ascorbate import in studies employing radiolabeled ascorbate.

Gap junction hemichannels

Gap junction hemichannel involvement has been suggested by connexin reconstitution studies in microsomal and liposomal systems in which posttranslational integration of connexin oligomers results in bidirectional ascorbate flux across the membrane (3 in Fig. 5) [110,111]. Though there is not yet any direct evidence that this pathway is operational in whole cells, the mechanism remains plausible as gap junction hemichannels have been observed to facilitate release of other small metabolites from cells including glutathione [119] and ATP [120]. Moreover, gap junction-mediated ascorbate transport may allow for direct transcellular transport of the vitamin between coupled cells in the mammalian lens [121]. Further molecular genetics and pharmacological studies are required to assess the involvement of these channels in cellular ascorbate release.

Glutamate–ascorbate heteroexchange

Uptake of the major mammalian excitatory neurotransmitter, L-glutamate, stimulates the neuroprotective [54] and neuromodulatory [122] release of ascorbate by both astrocytes [107] and neurons [54,104,112]. The originally proposed mechanism for this stimulation was one of glutamate–ascorbate heteroexchange [112], in which glutamate uptake is biconditionally dependent on ascorbate release (4 in Fig. 5). As recently reviewed by Harrison and May [55], a growing number of experimental observations suggests that this model is incorrect. A further argument against this model is that glutamate-stimulated release of ascorbate demonstrates an apparent affinity for glutamate that suggests involvement of one or more glial sodium-dependent excitatory amino acid transporters (EAATs) [107]. The activity of these transporters is known to be sodium dependent but not dependent on heteroexchange with ascorbate. The involvement of these transporters in ascorbate efflux from brain cells, however, remains to be directly demonstrated.

Ascorbate release by exocytosis

Secretory granules from rat parotid gland (an exocrine tissue) contain approximately 3 mM ascorbate [113,123], while secretory vesicles from endocrine tissues such as pituitary [124] and adrenal medulla [125–127] contain ascorbate concentrations as high as 20 mM. Accumulation of ascorbate in these vesicles appears to depend on expression of SVCT2, at least in the adrenal medulla [128]. Upon plasma membrane fusion of these secretory vesicles, intraluminal ascorbate is released to the extracellular space (5 in Fig. 5). In the case of the adrenal medulla, exocytotic ascorbate release into the bloodstream is regulated by nicotinic acid receptor activation in a Ca^{2+}-dependent manner [125]. This suggests that exocytotic ascorbate release occurs by a form of regulated, rather than constitutive, exocytosis. The most studied form of Ca^{2+}-regulated exocytosis is that of neurotransmitter release by presynaptic neurons [129] and gliotransmitter release by astrocytes [130,131]. In the case of astrocytes, gliotransmitter release includes the regulated exocytotic efflux of small signaling molecules such as L-glutamate, ATP, and D-serine (reviewed by Montana and colleagues [131]).

Astrocytes in culture are known to release large amounts of ascorbate following intracellular ascorbate loading with either DHA or ascorbate [35,107,116,132,133]. As reviewed below, this release is vital for maintenance of neuronal ascorbate and neuroprotection. The precise mechanisms of this release remain ill-defined. Though VSOACs and gap junction hemichannels are likely to contribute to some of this release [107,116], a large proportion of ascorbate release remains unaccounted for by these mechanisms. The contribution of regulated exocytosis to general gliotransmitter release by astrocytes suggests that it may also contribute to ascorbate release by these cells. The involvement of exocytosis in ascorbate release by cells in general requires further investigation.

Recent studies with endothelial cells—A role for $P2Y_2$ receptors

A recent report by May and Qu [118] demonstrates that ascorbate release by EA.hy296 endothelial cells is not inhibited by generic anion channel inhibitors and is nonsaturable with respect to the intracellular ascorbate concentrations achieved. Moreover, a significant proportion of ascorbate release by these cells is also stimulated by Ca^{2+} entry into cells, as previously documented for coronary artery endothelial cells [102]. In the latter study, ATP also stimulated Ca^{2+}-dependent ascorbate release by acting on $P2Y_2$ G-protein-coupled purinergic receptors. Interestingly, both studies conclude that ascorbate efflux in endothelial cells probably occurs via diffusion across channels in the plasma membrane [102,106,118]. However, these data may also be explainable by an exocytotic mechanism. For example, $P2Y_2$ receptor

activation has been observed to stimulate primary granule release from human neutrophils [134], suggesting that $P2Y_2$-mediated, Ca^{2+}-dependent exocytosis could plausibly contribute to ascorbate release. The question of whether or not this actually occurs remains to be answered.

Physiological functions of ascorbate release—Recent insights

Though ascorbate release from cells is an indisputable phenomenon, the physiological functions performed by this release remain largely unknown. At the very least it can be said that the release of ascorbate by cells in mammalian systems must be relevant to overall body ascorbate homeostasis, including enterocytic release of ascorbate following intestinal absorption; hepatic release of ascorbate following biosynthesis in ascorbate-producing species; release of ascorbate back into the blood stream following glomerular reabsorption in the kidney; release of ascorbate to the interstitial space after endothelial uptake from plasma [118]; and release of ascorbate to the cerebrospinal fluid by choroid plexus epithelial cells following uptake from the plasma [55]. Several recent reports suggest that export of ascorbate may contribute among other things to (i) the replenishment of extracellular ascorbate in various tissues [62,106], (ii) to transcellular electron transfer between astrocytes and neurons in the brain [53,54,104,132], and (iii) to the reduction of non-transferrin-bound iron prior to cellular uptake [77,85,105]. Only the latter three functions will be discussed here, as these examples serve to illustrate the diversity of the functions achieved by ascorbate efflux.

Ascorbate release and extracellular ascorbate replenishment—A role for erythrocytes?

In theory, ascorbate release by cells can allow for the replacement of extracellular ascorbate that has been oxidized or degraded, at the expense of intracellular ascorbate [106]. Though yet to be demonstrated as a general phenomenon, this replacement is likely to be an important contributor to overall ascorbate homeostasis in extracellular fluids in mammals. This point is particularly pertinent to humans and other ascorbate auxotrophs, and it may be anticipated that this form of ascorbate regeneration may help compensate for the lack of endogenous ascorbate production. For example, it has been calculated that human erythrocytes have the capacity to regenerate total blood ascorbate over every 3 min [47]. Presumably, a large proportion of this recycling capacity comes from transplasma membrane ascorbate:AFR oxidoreduction [47,48], as discussed earlier. However, as human erythrocytes can release significant amounts of ascorbate (up to one-fifth of intracellular ascorbate over 40 min, or 50% over 2–3 h) [47], and appear to be biochemically adapted to import and recycle DHA back to ascorbate intracellularly [62–66], this release may also help contribute to overall plasma ascorbate levels. Even though fractional ascorbate release rates from erythrocytes are somewhat lower than most nucleated cells examined—which in light of the above discussions may be a consequence of a lack of exocytosis—the sheer volume of the overall erythrocyte population, as well as their favorable surface area:volume ratio, could provide for an efficient and mobile means of ascorbate regeneration within the body (Fig. 6A). Despite these speculations, the relative contributions of transplasma membrane AFR reduction and ascorbate release to maintenance of plasma ascorbate by erythrocytes remain to be thoroughly examined.

Astrocytes supply neurons with a ready source of ascorbate

In addition to glutathione, ascorbate is a major brain antioxidant and is present in this tissue at millimolar concentrations [54,112]. Though brain ascorbate is predominantly derived from the plasma by SVCT2-dependent transport across the choroid plexus [54,55], astrocytes are central to the maintenance and dynamic regulation of brain ascorbate [35,132]. Astrocytes are far more resistant to oxidative stress than neurons and play key roles in determining the susceptibility of neurons to oxidative damage [132,135]. As well as indirectly supporting neuronal glutathione metabolism, astrocytes bolster neuronal antioxidant status by supplying the neurons they flank with a ready supply of ascorbate. Neuronal ascorbate levels are

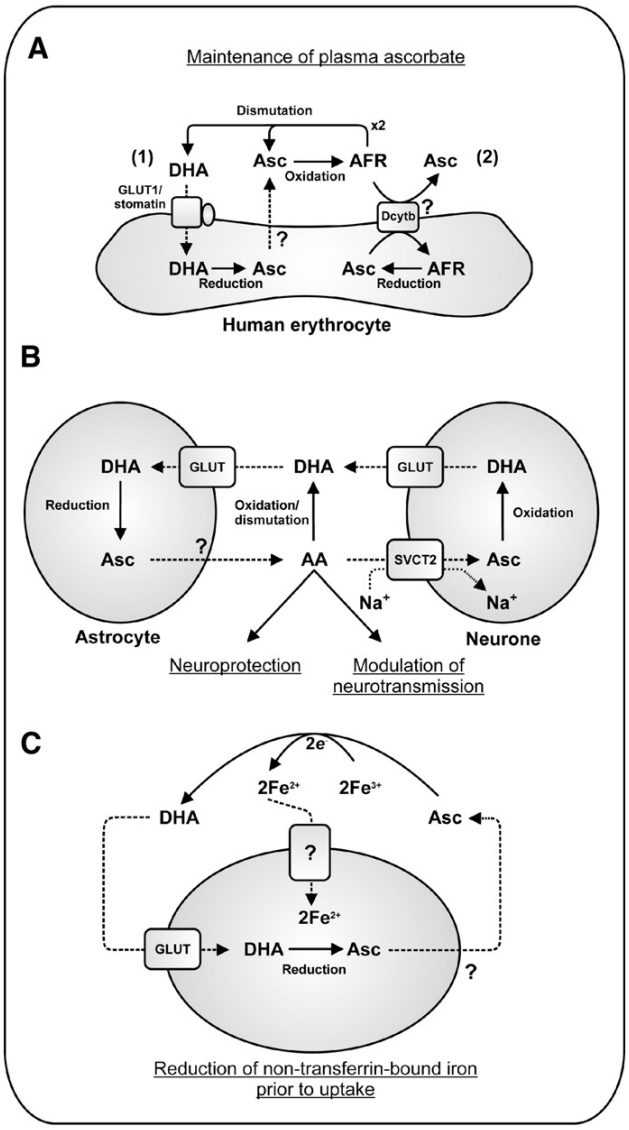

Fig. 6. Functions of ascorbate efflux in blood and brain tissue and iron uptake. (A) Human erythrocytes may be adapted to regenerate ascorbate (Asc) that is oxidized in plasma by at least two mechanisms: (1) the slow release of Asc that is formed as a result of glucose-insensitive DHA import by the GLUT1/stomatin complex and intracellular DHA reduction; and (2) enzymatic, ascorbate-dependent tPMET to extracellular AFR by an oxidoreductase such as Dcytb. (B) Astrocytes help to protect neurons from oxidative damage in the brain, in part by providing neurons with a ready supply of Asc released from the astrocyte interior. Neurons import this Asc in an SVCT2-dependent manner. Astrocytes additionally import neuronally released DHA (in a GLUT-dependent manner) following intraneuronal Asc oxidation, and reduce it intracellularly. This Asc cycling constitutes a crucial redox "coupling" between these two cell types, representing a form of transcellular electron transport in which efferent tPMET within astrocytes (at the expense of astrocytic metabolism) is coupled to afferent tPMET within neurons. (C) Cells can also release Asc that has been formed intracellularly as a result of DHA import and subsequent reduction. This Asc can react quantitatively with extracellular non-transferrin-bound ferric iron, reducing it to ferrous iron that can be imported by ferrous-selective transporters [85]. As cells are typically bathed in ascorbate concentrations that are more than sufficient to reduce typical levels of this form of iron, this mechanism illustrates the more general notion that extracellular ascorbate regeneration by cells may contribute to iron reduction prior to uptake. In all cases (i.e., A–C), the mechanisms of Asc efflux remain to be established.

approximately 10-fold higher than those of astrocytes [54], which may be due to lack of in vivo SVCT2 expression in astrocytes [136]. The higher requirement for ascorbate in neurons compared with astrocytes is justified in part by their 10-fold higher rates of oxidative metabolism and susceptibility to excitotoxicity and oxidative stress. The ascorbate released in the vicinity of neurons is employed extracellularly in neuromodulation of glutamatergic and dopaminergic signaling [122], and provides antioxidant protection against glutamate-induced excitotoxicity and oxidative stress [54,112,137–141].

Additionally, astrocytes import and reduce DHA that is released by neurons following intraneuronal ascorbate oxidation [35,116,132,135,142]. Efficient uptake of DHA by astrocytes is important for neuroprotection because DHA is neurotoxic [143], but not gliotoxic [144]. Together these processes constitute an efficient ascorbate cycle between astrocytes and neurons (Fig. 6B) that is vital to neuronal function and antioxidant status [135,145].

Ascorbate release and cellular iron uptake

The release of ascorbate by cells has also been implicated as an additional, nonenzymatic mechanism that cells may use to reduce non-transferrin-bound iron prior to uptake by ferrous-selective transporters [77,85,105]. In the most recent of these studies [85], it was demonstrated that ascorbate released from K562 cells—following uptake and reduction of DHA—mediates direct reduction of ferric to ferrous iron, the latter of which is then imported (Fig. 6C). Importantly, stimulation of ferrireduction by ascorbate was entirely attributable to ascorbate release from the cell interior. This suggests that ascorbate-dependent enzymatic tPMET to ferric iron (as discussed above) is not involved in non-transferrin-bound iron uptake by K562 cells.

Interestingly, in silico modeling of iron speciation in human plasma [146] suggests that in the presence of physiological ascorbate concentrations, non-transferrin-bound iron exists largely as low molecular weight ferrous complexes. Therefore, processes that maintain extracellular ascorbate—such as ascorbate release and transplasma membrane AFR reduction—may be expected to contribute substantially to iron reduction and uptake in vivo. Moreover, the observation that ascorbate released into the lumen of the digestive tract contributes to dietary nonheme iron absorption [99,105,147,148] further supports the general mechanism of direct chemical reduction of low molecular iron chelates prior to ferrous-selective uptake across cell membranes. Importantly, the relative contributions of ascorbate release and enzymatic ferrireduction mechanisms (e.g., that attributed to Dcytb) [12,70,149,150] are yet to be established.

Conclusions

In this review we have explored the notion that ascorbate contributes to cellular physiology in large part through ascorbate-dependent electron transfer across the plasma membrane. We have suggested that the mechanisms of this transfer are probably twofold, consisting of (i) classical enzymatic electron transfer through the redox centers of plasma membrane ascorbate oxidoreductases (e.g., Dcytb), and (ii) nonclassical transplasma membrane ascorbate export by plasma membrane conduits and/or exocytosis. As intracellular ascorbate is strongly maintained at the expense of cellular metabolism, both forms of ascorbate-dependent tPMET result in the net transfer of intracellular reducing power to the extracellular space for deployment in a variety of disparate, but ultimately redox-dependent processes. These processes include the maintenance of ascorbate concentrations in plasma and interstitial fluids such as that of the brain, the transcellular transport of reducing equivalents from astrocytes to neurons, and the reduction of iron prior to uptake. As discussed, several of these processes probably utilize both forms of ascorbate-dependent tPMET. There are still many unknowns concerning the precise redox mechanism and physiological acceptors of Dcytb-dependent electron transfer from intracellular ascorbate across the plasma membrane, and concerning the mechanisms and regulation of ascorbate efflux from cells. In conclusion we suggest that both enzyme- and efflux-based ascorbate-dependent electron transfers should be included as alternative modes of ascorbate-dependent tPMET.

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