Transplasma membrane electron transport comes in two flavors

Darius J. R. Lane and Alfons Lawen*

Department of Biochemistry and Molecular Biology, School of Biomedical Sciences, Monash University VIC 3800, Australia

Abstract. All tested cells possess transplasma membrane electron transfer (tPMET) systems that are capable of reducing extracellular electron acceptors at the cost of cytosolic electron donors. In mammals, classically NAD(P)H- and NADH-dependent systems have been distinguished. The NADH-dependent system has been suggested to be involved in non-transferrin-bound iron (NTBI) reduction and uptake. Recently we reported that transplasma membrane ascorbate/dehydroascorbate cycling can promote NTBI reduction and uptake by human erythroleukemia (K562) cells (D.J.R. Lane and A. Lawen, J Biol Chem 283 (2008), 12701–12708). This system, involves i) cellular import of dehydroascorbate, ii) intracellular reduction of dehydroascorbate to ascorbate using metabolically-derived reducing equivalents, iii) export of ascorbate down its concentration gradient, iv) direct reduction of low molecular weight iron chelates by ascorbate, and v) uptake of iron (II) into the cell. We here propose the consideration of this system as a novel form of tPMET which shares with classical enzyme-mediated tPMET systems the net transfer of reducing equivalents from the cytoplasmic compartment to the extracellular space, but lacks the involvement of the plasma membrane oxidoreductases responsible for the latter. Thus, transplasma membrane electron transfer can and does occur at two mechanistically distinct levels: i) enzyme-mediated transmembrane electron transfer and ii) transmembrane metabolite shuttling/cycling.

Keywords: Astrocytes, dehydroascorbate, K562 cells, non-transferrin-bound iron, Vitamin C

Abbreviations: AFR, ascorbate free radical; DHA, dehydroascorbate; GLUT, facilitative glucose transporter; NTBI, non-transferrin-bound iron; tPMET, transplasma membrane electron transport; SVCT, sodium-ascorbate co-transporter; VSOAC, volume-sensitive osmolyte and anion channel

1. Introduction

Transplasma membrane electron transport (tPMET) in eukaryotes is now well established [8,10,15,44, 59,61,68]. The concept of tPMET arose from the observation that cell-impermeant dyes [43,87] can be reduced by tissue slices [97]. tPMET activities have since been related to the regulation of vital cellular processes including cellular bioenergetics [53,86], growth control and differentiation [8,15,68], apoptosis [54,55,70,101], pH control and mitogenesis [8,68], cell signal transduction [68], antioxidation [61, 85], and iron/copper metabolism [8,15,67,68,102]. Accordingly, deregulation of tPMET has been linked to various human conditions including aging and neurodegeneration [36,37], macrophage-mediated LDL oxidation in atherogenesis [9], diabetic nephropathy [60] and glycolytic cancer progression [31–33].

*Address for correspondence: Alfons Lawen, Department of Biochemistry and Molecular Biology, School of Biomedical Sciences, Monash University, VIC 3800, Australia. Tel.: +61 3 9905 3711; Fax: +61 3 9905 3726; E-mail: alfons.lawen@med.monash.edu.au.

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Classically a distinction was made between NAD(P)H- and NADH-dependent systems, the former of which includes the members of the Nox and Duox families [59], while the latter – often referred to as the plasma membrane NADH:oxidoreductase system or PMOR – is suggested to include at least an NADH oxidase and an NADH:ferricyanide reductase activity [8,15,59].

Several enzymes have been suggested to be responsible for the plasma membrane NADH:ferricyanide reductase activity: a 57 kDa NADH-quinone oxidoreductase from rat liver plasma membranes [46]; a plasma membrane localized voltage-dependent anion channel (VDAC) isoform 1 [7]; and a membrane-bound form of cytochrome \( b_5 \) reductase in neuronal plasma membranes [79]. Moreover, a multi-component, quinone-dependent tPMET system is also well-described [31] that is capable of reducing cell-impermeant water-soluble tetrazolium salts (e.g. WST-1) or extracellular dioxygen at the expense of intracellular NADH [10,31].

Iron is vital for cellular survival: without it, every cell will die. Iron is a cofactor for oxidative phosphorylation, neurotransmitter and nucleotide synthesis, nitric oxide metabolism and oxygen transport. However, iron can also catalyze the formation of reactive oxygen species [21,75,103]. Since too much and too little iron can compromise cell viability, cellular iron homeostasis has to be tightly controlled.

In its physiological form, extracellular iron is complexed by biological chelators, the most important of which are transferrin and citrate. Whereas the uptake of iron from transferrin is reasonably well understood, the mechanism of non-transferrin-bound iron (NTBI) uptake by mammalian cells remains elusive. In order for iron citrate to be taken up by a cell, iron has to be first reduced from iron (III) to iron (II) as almost all cellular iron uptake can be inhibited by iron (II) chelators [18,30,39,41,76,91]. Ferrous iron is then taken up by ferrous-specific transporters (e.g., DMT1 [5,104] and Zip14 [58]) in the plasma membrane.

Soon after its discovery, the transplasma ferricyanide reductase activity was suggested to be responsible for the reduction of NTBI prior to uptake as Fe\(^{2+}\) through the plasma membrane by divalent metal ion transporters [3,15,17,39,41]. Several enzymes have been suggested to be responsible for NTBI reduction before uptake, including duodenal cytochrome \( b_{561} \) (Dcytb [67]) and voltage dependent anion-selective channel, isoform 2 (VDAC2 [94]). The involvement of Dcytb in NTBI uptake, however, has since been questioned [27] and work in our own laboratory was not successful in linking either VDAC1 or VDAC2 to NTBI uptake.

Ascorbate is known to promote the bioavailability of iron from numerous food sources in vivo and in vitro [22,29,30]. An ascorbate-stimulated ferricyanide reductase has been described, but remains to be molecularly identified. Moreover, ascorbate supplementation was shown to stimulate extracellular ferricyanide reduction by several cell types, including K562 cells [51,82,83], HL-60 cells [95] and human erythrocytes [44,61]. These data prompted us to ask the question of whether the ascorbate-stimulated ferricyanide reductase is involved in iron reduction for the uptake of iron from iron citrate.

### 2. The biochemistry of ascorbate

The physiologically prevalent monovalent ascorbate anion can undergo sequential one-electron oxidations under physiological conditions of pH, temperature and oxygen tension [61,78] (Fig. 1). The first oxidation yields the relatively long-lived and electrochemically stable ascorbate free radical (AFR; also known as semi- or mono-dehydroascorbate; \( E'_0 = +330 \) mV) [72]. This first oxidation of ascorbate is enhanced by low levels of circulating redox-active transition metals, such as iron and copper [78,84,88,89]. As AFR is relatively unreactive with dioxygen [78] – unlike many other free radicals [13,57] – and tends to decay mainly by disproportionation [11], the formation of AFR by reaction of ascorbate with
Fig. 1. The oxidation products of vitamin C. The undissociated, fully reduced form of vitamin C (ascorbic acid) undergoes a monoprotic ionization at the carbon-3 hydroxyl with a $pK_a$ of 4.2, so that the ascorbate monoanion is the predominant species at physiological pH. Ascorbate can undergo a thermodynamically favorable and reversible one-electron oxidation to the ascorbate free radical (AFR). AFR is stabilized by resonant distribution of the resultant unpaired electron over the ring structure. AFR can undergo a subsequent reversible one-electron oxidation to form the two-electron oxidized form, dehydroascorbate (DHA). DHA is highly unstable under physiological conditions and undergoes an essentially irreversible hydrolytic ring opening to 2,3-diketogulonic acid (DKG) with a half-life of several minutes.

reactive radical species tends to inhibit free radical-induced oxidative chain reactions [13,57]; especially in the face of rapid AFR reduction back to ascorbate. AFR can undergo a further monoelectronic oxidation to DHA ($E'_0 = -210$ mV) [72] in the presence of mild oxidants such as ferricyanide [61,96] and/or NTBI species [14,88,89]. In the absence of such oxidants, however, two AFR molecules will rapidly disproportionate to one ascorbate and one DHA molecule [11,63] (Fig. 1). Though oxidation (or disproportionation) of AFR to DHA effectively allows utilization of the two-electron reducing capacity of ascorbate, DHA is a structurally labile species that rapidly undergoes an irreversible hydrolytic ring-opening to 2,3-diketogulonic acid under conditions found in plasma with a half-life of several minutes [48,49].

Degradation of DHA results in an irrevocable loss of the vitamin from mammalian systems [42, 48,49] – a point that is particularly pertinent in the case of species lacking gulono-$\gamma$-lactone oxidase activity [92]. In order to cope with this tendency for ascorbate loss, the vitamin must be maintained predominantly in the two-electron reduced form (i.e. ascorbate) in both intra- and extracellular biological fluids [74]. Consistent with this observation, mammalian cells possess a variety of conservative reductive mechanisms for maintaining both intra- and extracellular ascorbate [61,66,78,99]. Even cultured cells – which may be chronically ascorbate-deficient due to lack of supplementation under standard culture conditions [35,90] – still maintain an extraordinary ability for ascorbate regeneration.

3. The ascorbate-stimulated plasma membrane ferricyanide reductase

Human erythrocytes possess a tPMET activity that utilizes intracellular ascorbate as the major electron donor to reduce extracellular ferricyanide [44,61]. It is not clear at this stage whether ascorbate is a
Fig. 2. DHA uptake by K562 cells occurs by GLUT-mediated transport. To assess the involvement of facilitative glucose transporters in DHA uptake by K562 human erythroleukemia cells, cells previously grown to \(6 \times 10^6\) cells/ml in RPMI + 10% fetal bovine serum at 37°C, 5% CO2 and 95% air were initially washed three times with MOPS-buffered saline (MBS, 137 mM NaCl, 2.7 mM KCl, 15 mM MOPS-Na+, pH 7.3). (a) Washed cells were then exposed to increasing concentrations of the GLUT-inhibitor cytochalasin B (CB) or the non-GLUT inhibiting structural analog, dihydrocytochalasin B (H2CB) 10 min prior to, and during incubation with 400 \(\mu\)M dehydroascorbate (DHA) for 30 min at 37°C. Cells were then washed three times in 100 volumes in cold MBS and their intracellular ascorbate determined essentially according to Lane and Lawen [52]. As DHA uptake is inhibitable by CB, but not H2CB, GLUT-mediated DHA uptake is implied. (b) Alternatively, washed cells were exposed to increasing concentrations of the GLUT-transportable, but non-metabolizable glucose analog 3-O-methyl-D-glucose (3-OMG) or the non-GLUT-transportable glucose stereoisomer L-glucose prior to incubation with DHA as in panel A. Again the results indicate GLUT involvement in DHA import as inhibition of intracellular ascorbate accumulation occurs only in the presence of the GLUT-transportable glucose analog.

4. Cellular DHA uptake

The majority of mammalian cells maintain intracellular ascorbate concentrations that are markedly higher (e.g. up to 30-fold in some cases [47]) than those in the extracellular fluid or plasma [61, 78,99]. Though many cells maintain this outward-facing concentration gradient by SVCT-mediated ascorbate import [81,99], the facilitated diffusion of DHA through low-affinity, high-capacity GLUTs is also a significant contributor [99]. With respect to DHA uptake by cells, an inward-facing GLUT 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 [34,77,99]. K562 cells showed elevated intracellular ascorbate levels after loading with DHA [52] that was inhibitable by cytochalasin B, suggesting response-dependence on DHA uptake via GLUTs, as previously suggested [77,78,93,99]. The involvement of GLUTs in DHA uptake by K562 cells is further supported by two pharmacological observations that intracellular ascorbate accumulation in response to extracellular DHA is inhibited by: i) low micromolar
Fig. 3. Ascorbate/DHA shuttling in mammalian NTBI uptake. Recent evidence suggests that NTBI ferrireduction may occur by transplasma membrane Asc cycling in which i) extracellular Asc reacts directly with NTBI, forming both DHA and Fe$^{2+}$. The latter is then imported into the cell putatively via ferrous-selective transporters (e.g. DMT1 and/or Zip 14). Extracellular Asc is subsequently regenerated for further ferric reduction events by ii) DHA import via glucose transporters (GLUTs), iii) intracellular reduction of DHA to Asc by an unspecified redox couple (’R/O’; e.g. GSH/GSSG or NADPH/NADP$^+$), followed by release of Asc through as yet unidentified Asc transporters (Anion Channel) in the plasma membrane (PM).

concentrations of cytochalasin B, but not the structural analog dihydrocytochalasin B (Fig. 2a), the latter of which shares with cytochalasin B its inhibition of cellular motile processes but not that of facilitated glucose transport [56]; and ii) millimolar concentrations of the transportable (but non-metabolizable) D-glucose analog 3-O-methyl-D-glucose, but not the non-transportable glucose stereoisomer L-glucose (Fig. 2b). Again, primary astrocytes demonstrate similar behavior (Lane et al., data not shown).

5. Iron uptake and the ascorbate/DHA shuttle

Cellular uptake of NTBI is well documented, but less well understood than the classical transferrin-dependent iron import pathway [2,16,19,28,38,39,41,50,64,71]. The former may be particularly relevant in iron overload diseases such as hereditary hemochromatosis, hypotransferrinemia, and thalassemia [4,12,18,23], in which plasma iron presents in excess of transferrin-binding capacity [6]. When we analyzed the ascorbate-mediated stimulation of NTBI reduction and uptake by human erythroleukemia (K562) cells we found that DHA loading of cells stimulated both processes (viz. 12- and 2-fold, respectively), yet – unlike the reduction of ferricyanide – remained inhibitable by extracellular ascorbate oxidase [52]. Furthermore, as cells were able to import iron in a manner inhibitable by cell-impermeant ferrous ion chelators, the ascorbate-stimulated iron uptake is clearly dependent on the initial adoption of the ferrous state, as previously observed [18,30,38,39,41,69,76,91].

Our data suggest that ascorbate released from cells – following uptake and reduction of DHA – mediates direct reduction of ferric to ferrous iron, the latter of which is then imported (Fig. 3). Subsequent addition of DHA to control or loaded cells resulted in a dose-dependent stimulation of both iron reduction and
uptake that was inhibitable by cytochalasin B, suggesting response-dependence on DHA uptake via GLUTs (see Section 4). Again, these results are basically reproducible with primary astrocyte cultures (Lane et al., data not shown).

Several plausible candidates for the cellular export of ascorbate have been proposed [99], including exocytosis of ascorbate-containing vesicles [98,99], ascorbate-ascorbate homeoexchangers [24,45], connexin hemi-channels [1] and volume-sensitive osmolyte and anion channels (VSOACs) [47,62,99,100]. VSOAC permeability [80] and ascorbate efflux from cells [47,99] can be inhibited by generic anion channel inhibitors, such as 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) and 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS), suggesting that a significant proportion of ascorbate release occurs via this pathway. The observation that DIDS inhibits ascorbate release, ferrireduction and iron uptake to a similar degree in K562 cells [52] supports this conclusion.

6. ‘Shuttle-based’ tPMET systems – conclusions

Historically, research focused on enzymatic tPMET systems; however several examples of ‘shuttle-based’ tPMET systems have been documented as well, including ascorbate/DHA [30,52,64], dihydrolipoic acid/α-lipoic acid [40,65], reduced glutathione/cysteine [20,25] and superoxide/dioxygen [26,73] shuttles. As with classical enzyme-mediated tPMET systems, these ‘shuttle-based’ systems result in the net transfer of metabolically-derived reducing equivalents from the cytoplasmic compartment to the extracellular space. Once in the extracellular space, however, the fate of these reducing equivalents will depend on the particular redox couple involved. A canonical example of shuttle-based tPMET is transplasma membrane ascorbate/DHA cycling, which was the focus of this brief review. Our suggestion is that tPMET can and does come in two ‘flavours’: i) enzyme-mediated transmembrane electron transfer and ii) transmembrane metabolite shuttling/cycling. In the latter reducing equivalents derived from cellular metabolism are transferred – via transmembrane metabolite shuttling – to the extracellular space for participation in extracellular redox events. Transplasma membrane ascorbate/DHA cycling may contribute significantly to NTBI ferric reduction prior to ferrous uptake.

References


[52] D.J.R. Lane and A. Lawen, Non-transferrin iron reduction and uptake are regulated by transmembrane ascorbate cycling in K562 cells, J Biol Chem 283 (2008), 12701–12708.


