ANTIOXIDANTS & REDOX SIGNALING
Volume 2, Number 2, 2000
Mary Ann Liebert, Inc.

Forum Review Article

Functions of Vitamin C as a Mediator of Transmembrane Electron Transport in Blood Cells and Related Cell Culture Models

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ABSTRACT

Vitamin C (ascorbic acid) is an important physiological antioxidant. Within cells, it is practically always present in the reduced form. Several enzymatic and nonenzymatic mechanisms have been reported to maintain this status. In the extracellular environment, oxidation of ascorbate leads to loss of vitamin because the oxidized form, dehydroascorbic acid, is unstable under physiological conditions. The intermediate ascorbate free radical, although rather long-lived for a free radical, quickly disproportionates into the two other forms, also leading to loss of vitamin. Protection from loss can only be achieved by cellular regeneration mechanisms, *i.e.*, by uptake of dehydroascorbic acid and either storage or recycling, and by plasma-membrane mediated reduction of extracellular free radical or dehydroascorbic acid. Moreover, intracellular ascorbate can also serve as an electron donor for transmembrane reduction of external electron acceptors. However, the physiological significance of this function is as yet unknown. The results presented in the literature are sometimes conflicting as to the relative contributions of these different possibilities, which seem to differ in different cell types. In this short review, the various pathways of regeneration of ascorbate and their relative contributions to the avoidance of vitamin loss in plasma or cell culture medium are discussed. Antiox. Redox Signal. 2, 189–196.

INTRODUCTION

Ascorbate (vitamin C) is known as an important antioxidant as well as a necessary cofactor for a number of metabolic reactions such as biosynthesis of collagen, carnitine, norepinephrine, and a number of neuroamidopeptides. Although it is a significant factor in the antioxidative capacity of the plasma, its main place of action is the cellular cytosol and, indirectly, the cell membrane. Many cells accumulate ascorbate against a concentration gradient. Besides this movement of the molecule, which can occur in the reduced form ascorbate as well as in the oxidized form dehydroascorbic acid, there is also apparent communication between the antioxidant pools of the intracellular and the extracellular milieu by way of electron transport through the membrane, at least in blood cells (erythrocytes as well as leukocytes) and tumor cells of myeloid origin. This is possible by the vitamin as electron carrier itself, a process termed ascorbate recycling, but also by ascorbate-mediated electron transfer. This again has been observed with the oxidized forms of ascorbate dehydroascorbic acid and, more frequently, ascorbate free radical (mono- or semidehydroascorbate) as extracellular electron acceptors as well as with ascorbate as the internal electron donor.

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In principle, this could also result in an electron shuttling from the intracellular to the extracellular pool, such as is observed in the chromaffin granules of the adrenal medulla, but this has not been described yet.

By reviewing existing data and presenting some unpublished results, this review tries to differentiate the relative contributions these different mechanisms operating in blood cells and experimental cell culture models make to avoid losses in vitamin. These are largely due to the inherent instability of dehydroascorbic acid (DHA) (Borsook *et al.*, 1937) and are the main reason for the daily requirement of 60 odd milligrams (Levine *et al.*, 1996).

PLASMA AND ERYTHROCYTES

Vitamin C recycling

The concentration of ascorbate in plasma is around 60 μ *M* (Wang *et al.*, 1992). Because almost half of the blood volume is occupied by the red blood cells, one would expect these cells to contain either an equal concentration as the plasma itself or a higher one due to active uptake and storage.

Indeed, the erythrocytes are the only cells that release ascorbate to a significant extent and therefore do not store it. It has been known for many years, that these cells accumulate oxidized ascorbate, *i.e.*, dehydroascorbic acid, much more efficiently than the reduced form (Hughes, 1964; Rose, 1988; Goldenberg and Schweinzer, 1994). This is now established for many other cells and tissues and is considered a general mechanism, but the molecular understanding of this property has only recently been understood (see below). A recycling pathway with the effect of exporting reducing equivalents from the cells into the plasma was first described 20 years ago (Orringer and Roer, 1979). It clearly demonstrated that cells have the ability to keep ascorbate in the reduced form, to reduce it after uptake of DHA (Mann and Newton, 1975). This has been confirmed by recent studies, and glutathione has been identified as the main electron donor for this reduction (Winkler, 1992; May *et al.*, 1996b, 1997). As outlined below, this antioxidant is generally responsible for this reduction, but it is not absolutely necessary in other cell types.

Monodehydroascorbate reductase and ascorbate-ferricyanide reductase

Besides their ability to recycle ascorbate, erythrocytes have also been shown to contain the enzyme NADH-monodehydroascorbate reductase in their membranes (Goldenberg *et al.*, 1983). This enzyme is associated with the outer mitochondrial membrane in most of the tissues in which it has been identified, where it serves as a means of regeneration of ascorbate from the ascorbate free radical formed by reactions such as the transmembrane reduction of catecholamines in adrenergic neurons (Diliberto *et al.*, 1982; Levine *et al.*, 1983). In intact red blood cells, where the only possible membrane location obviously is the cell membrane, the transplasma membrane topology of this activity was first demonstrated by the ability of DHA and ascorbate to stimulate the reduction of extracellular ferricyanide independently of ascorbate release (Schipfer *et al.*, 1985). Later, the mechanism of this stimulation was shown not to be due to this enzyme activity, but to be directly connected to the transport of DHA into the cells. Apparently, the transmembrane electron transfer responsible for the reduction of the iron complex draws the electrons from intracellular ascorbate accumulated by the transport-reduction coupling (May *et al.*, 1995a). The mechanism could be simulated in ghosts resealed with ascorbate (May *et al.*, 1996a). The identity of the two processes is also indicated by their kinetic likeness. In both experimental setups, the maximal velocity of ferricyanide reduction was around 2 $pmol \cdot min^{-1} \cdot 10^{-6}$ cells. This is very little compared to rates found in cells which actively store ascorbate against a concentration gradient. Supplementation of the membrane with α-tocopherol (vitamin E) enhances the electron transport rate (May *et al.*, 1996a, 1998). Ascorbate can regenerate α-tocopherol (Packer *et al.*, 1979), and this can be oxidized by external electron acceptors. Thus, one would expect this stimulation, but whether it is caused by the same enzymatic activity (if by any at all) is not certain (May, 1999).

Consequences of ascorbate function in erythrocytes for plasma antioxidant capacity

Release of ascorbate from erythrocytes can also contribute to the antioxidant pool of the plasma. Although the release rates are rather small (Hughes and Maton, 1968), the contribution seems to be quite significant (Beyer, 1994; May *et al.*, 1995b; Mendiratta *et al.*, 1998; May, 1999). Interestingly, the release of ascorbate is significantly increased in malaria parasite-infected erythrocytes (test on mice, but possible also in humans; Iheanacho *et al.*, 1993). However, a physiological or pathological meaning of this observation has not been found yet; it may be just a by-effect of increased membrane permeability.

LEUKOCYTES AND RELATED TUMOR CELLS

Establishment of the outward concentration gradient by transport of dehydroascorbic acid and intracellular reduction

The situation is much more controversial in leukocyte type cells than in the comparatively simple red blood cells. Leukocytes store vitamin C against very high concentration gradients. The intracellular concentrations found in immunocompetent cells, particularly those which respond to external stimuli by activation of the respiratory burst, are in the millimolar range, up to 40-fold higher than in their environment (Washko *et al.*, 1989; Bergsten *et al.*, 1990; Goldenberg and Schweinzer, 1994; Bergsten *et al.*, 1995; Laggner *et al.*, 1999). Speculations and experimental evidence that uptake of DHA takes place via glucose transporters have been around for many years (Bigley *et al.*, 1983). It was also clear that glutathione plays a significant role in keeping the intracellular vitamin pool strictly in the reduced form. Whether this occurs by enzymatic or by nonenzymatic reduction is still somewhat controversial (Winkler, 1992), although the efficiency of this balance and the speed of its achievement argues rather for the enzymatic mechanism (Hughes, 1964; Washko *et al.*, 1993; Vera *et al.*, 1994, 1995; Welch *et al.*, 1995). Moreover, DHA reductases have been proven to exist in some of these cells, though not in all (Wells *et al.*, 1990, Park and Levine, 1996, May *et al.*, 1997). There is a wealth of literature on dehydroascorbate reductases in a number of different tissues, the citations are just a small choice closely related to the matter of transport. The transport mechanisms, however, are now clear beyond reasonable doubt. Glucose carriers of the GLUT-family, particularly the GLUT-1, which is the type mainly expressed in leukocytes, is also the carrier of DHA (Vera *et al.*, 1994; Rumsey *et al.*, 1997).

Implications for the primary immune response

A link between the respiratory burst and vitamin C transport can be drawn and has been experimentally demonstrated, which makes physiological sense because it enlarges the intracellular pool of the antioxidant under conditions of high oxidative stress (Washko *et al.*, 1993; Laggner and Goldenberg, 2000). But, of course, this logic only works if ascorbate in the environment of activated cells is really oxidized *in vivo* during the burst reaction. In resting cells, which also have high concentrations of ascorbate, the main transport pathway is a sodium dependent (Welch *et al.*, 1995) and was meanwhile demonstrated to occur via the sodium dependent vitamin C transporter SVCT (Daruwala *et al.*, 1999; Tsukagushi *et al.*, 1999). Under normal physiological conditions, where the plasma concentration of DHA is almost zero or very low (Wang *et al.*, 1992), this is most probably the way the cells obtain their high levels of vitamin C, similar to what is found in cells using the vitamin as a cofactor for the biosynthesis of collagen or have an exceptionally high requirement of this antioxidant, like the retinal pigment (see review by Goldenberg and Schweinzer, 1994).

Our recent study (Laggner and Goldenberg, 2000) shows that in differentiated HL-60 cells, a myeloid tumor cell that can be activated to initiate the respiratory burst, there is a very efficient response of the transport system to burst stimulation. In contrast to *in vitro* oxidation of ascorbate with either ascorbate oxidase or ferricyanide, where a large part of the vitamin present gets lost before the cells can catch it, and in contrast to the situation generated when superoxide anions are produced *in vitro* by ad-

dition of xanthine and xanthine oxidase, where only a weak stimulation of vitamin C transport occurs, the respiratory burst causes a stimulation of the uptake of DHA via the GLUT-1 transporter without generating any detectable extracellular DHA. Also, the losses are very slight (only 10% in contrast to 30–40% in the *in vitro* system described above). There is an apparent coupling of respiratory burst and transport that prevents the losses and thus keeps the antioxidant pool in shape very efficiently. The uptake rate is increased about 10-fold, depending on the concentration of ascorbate in the medium. The maximal rates for DHA uptake are around several hundred $pmol \cdot min^{-1} \cdot 10^{-6}$ cells, and around 20 $pmol \cdot min^{-1} 10^{-6}$ cells for the reduced form via the sodium-dependent transporter.

Leukocytes do not need glutathione to keep vitamin C reduced, or at least much less of it than the normal physiological concentration (Guiaquil *et al.*, 1997; Laggner *et al.*, 1999; May *et al.*, 1999a). Other enzymatic mechanisms can replace this electron donor without any damage.

Enzymatic electron transfer across the membrane

Whereas the preferred uptake of DHA is more or less out of the discussion, controversy exists about which contribution, if any, transmembrane electron transport can make to the stabilization of the vitamin C pool, and how it works. Transmembrane monodehydroascorbate reductase has been demonstrated experimentally by stimulation of the reduction of ferricyanide in intact K562 cells, an erythroleukemic cell line, by ascorbate or DHA, whereby both forms were almost equally effective. The activity is dependent on the membrane potential and apparently dependent on the concentration of the free radical (Schweinzer and Goldenberg, 1992, 1993). However, this latter finding has to be viewed with care, since the concentration of the radical cannot simply be calculated from the equilibrium of the symproportionation reaction between ascorbate and DHA. In the presence of traces of transition metals, it is always higher, at least at concentrations of ascorbate below 2.5 m*M* (van der Zee *et al.*, 1998), which is very high in terms of physiology. On the other hand, it was undetectable in the presence of ferricyanide (van Duijn *et al.*, 1998a), which makes the interpretation of the earlier data even more problematic.

Nevertheless, it is clear that the stimulation of the reduction of extracellular ferricyanide by vitamin C in either form involves a transmembrane electron transfer step distinct from recycling of vitamin C, which, though existing, is far too slow to account for the reaction (Schweinzer and Goldenberg, 1992). The rates are about 100 $pmol \cdot min^{-1} \cdot 10^{-6}$ cells, thus in a similar range as the transport of DHA.

In HL-60 cells as well as in the monocytic U937 cells, a transmembrane activity similar to that in erythrocytes was demonstrated, which rested on the uptake of DHA and the subsequent transfer of electrons from the intracellularly accumulated ascorbate to extracellular ferricyanide (van Duijn *et al.*, 1998b; May *et al.*, 1999a). Similar findings were also reported for a line of bovine pulmonary endothelial cells (Merker *et al.*, 1998). The similarity is striking, but nevertheless one must of course be cautious in comparing the situation of the human, who needs ascorbate as a vitamin, and the ruminant, which can synthesize it by itself and has a completely different metabolism of carbohydrates.

The activity is about three- to four-fold higher than the one described above in K562 cells, and the apparent affinity for ascorbate is an order of magnitude lower (30 μM versus 3 μM; van Duijn *et al.*, 1998b). In any case, the physiological significance is not clear, because an extracellular oxidant is needed to generate the enzymatic activity, and, as described above for the respiratory burst, the *in vivo* conditions can be completely different. It is also interesting to note that this electron transport chain does not reduce other iron complexes such as ferric citrate (May *et al.*, 1999b), which might be thought of as a physiological function of the reductase for uptake of non-transferrin-bound iron (Toole-Simms *et al.*, 1991). This makes its significance even more puzzling.

Stabilization of extracellular ascorbate by cellular antioxidant functions

A more physiological situation can be expected by following the fate of ascorbate in a

cell culture medium in the absence and in the presence of cells (Navas *et al.*, 1994). It is apparent that the life time of the vitamin is significantly enlarged by the cells. In the case of HL-60 cells, the difference in the decay rates has been used to calculate an enzymatic activity of monodehydroascorbate reductase (Alcain *et al.*, 1991) . This seems logical because ascorbate is first oxidized to the relatively stable free radical by a one-electron reaction, and reduction of the radical by the cells would prevent further oxidation via disproportionation of the radical to ascorbate and DHA and degradation of the latter (Bielski, 1982). The enzyme is apparently different from the ferricyanide reductase activity, the usual marker for transmembrane reductases (Villalba *et al.*, 1993). The apparent activities reported are very high (10 times higher than for the ferricyanide redox cycle), but are hard to understand on the basis of enzyme kinetics, because they represent a difference of decay rates rather than a true reduction rate. Membrane antioxidants, particularly coenzyme Q, enhance this stabilizing activity (Gomez-Diaz *et al.*, 1997). It has also been connected to the respiratory burst, although only indirectly. A transient rise in the apparent enzyme activity was found after differentiation of HL-60 cells with phorbol ester (Buron *et al.*, 1993)

The autoxidation of ascorbate in aqueous solution is practically solely due to the presence of traces of transition metals (Buettner, 1988). Findings reporting that K562 cells can stabilize ascorbate probably by just chelating away these metal ions show that the situation is more complex than can be represented by a single electron transfer reaction (Schweinzer *et al.*, 1993; Goldenberg and Schweinzer, 1995). Moreover, these latter cells are also able to reduce extracellular DHA directly, parallel to the uptake. The reduction prevails at low concentrations of DHA because it has a lower rate (3 pmol $min^{-1} \cdot 10^{-6}$ cells at the half-maximal concentration), but a much higher affinity (7 μM) than the uptake (approximately 400 μM) (Schweinzer *et al.*, 1996).

The activity of monodehydroascorbate reductase as stabilizing factor for ascorbate has not been demonstrated in membranes. Because the putative enzyme molecule is not identified, there are no molecular (*e.g.*, immunological) data showing its localization in the plasma membrane. When membranes from HL-60 cells, prepared by the method of Schlemmer and Sirotnak (1995), were mixed with ascorbate, the vitamin was also stabilized against oxidation by copper ions (H. Landertshamer and H. Goldenberg, unpublished observations; data showing these results can be made available on request to the corresponding author). This stabilization was dependent on the amount of membrane protein present, but was independent of any reductants like NADH, the putative electron donor of the enzyme. There was no increase of the concentration of ascorbate when DHA was added, either. Because a mixture of DHA and ascorbate increases the concentration of the ascorbate free radical by symproportionation, one would expect an enzymatic generation of ascorbate by the activity of the monodehydroascorbate reductase. Only upon addition of huge amounts of the oxidized vitamin (20 mM), a slight increase could be observed. This, however, could be a result of degradation, because some decay products of DHA show absorption in the range around 250 nm (Sawamura *et al.*, 1994) and also generation of ascorbate itself by nonreductive decay of DHA (Jung and Wells, 1998).

In intact cells, a decrease in the rate of disappearance of extracellular DHA should be expected upon addition of ascorbate, if the enzyme is operating. In the absence of ascorbate, dehydroascorbate is degraded. The mixture should be stabilized by reduction of the radical formed by symproportionation. This can indeed be shown, but only with a very large amount of cells (more than 50 million).

CONCLUSIONS

Involvement of ascorbate, ascorbate free radical and DHA in the mediation of electron transport have been demonstrated experimentally in many ways, as outlined in this review. However, most of the data reported originate from phenomenological experimentation. What is lacking is a clear identification of molecules, particularly of enzymes and prosthetic groups associated with them, which can be as-

signed responsibility for transmembrane electron transport mediated or influenced by either form of vitamin C.

The recent identification of the transporters for vitamin C, namely GLUT1 or other members of the glucose transporter family, for DHA, and the sodium-dependent vitamin C transporter for ascorbate, show the way. Without definition of transmembrane electron transport mediation by vitamin C, there will always be controversial speculation leading nowhere.

ACKNOWLEDGMENT

The authors were supported by the "Herzfeldersche Familienstiftung."

ABBREVIATION

DHA, dehydroascorbic acid.

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Received for publication December 17, 1999; accepted February 23, 2000.

This article has been cited by:

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