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Extracellular Ascorbate Stabilization: Enzymatic or Chemical Process?

J. C. Rodríguez-Aguilera[1] and P. Navas[1]

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Ascorbate is stabilized in the presence of HL-60 cells. This stabilization has been questioned as a simple chemical effect. Further properties and controls about the enzymatic nature of this stabilization are described and discussed. Our results showed that cAMP derivatives and cAMP-increasing agents stimulated the ability of HL-60 cells to stabilize ascorbate. On the other hand, tunicamycin, a glycosylation-interfering agent, inhibited this ability. These data, together with hormonal regulation, support the hypothesis of an enzymatic redox system located at the plasma membrane as being responsible for the extracellular ascorbate stabilization by HL-60 cells.

KEY WORDS: Ascorbate; redox; cAMP plasma membrane; HL-60.

INTRODUCTION

The importance of ascorbate in healthy organisms has been extensively reviewed (Padh, 1990; Gershoff, 1993). Ascorbate is one of the most important water-soluble free radical scavengers, and participate in collagen synthesis (Padh, 1990). Ascorbate can be synthesized by plants and most animals. However, rodents are the only mammals able to synthesize it. In all the others, humans among them, ascorbate is a dietary requirement acting as a vitamin (vitamin C). Long-term deprivation of ascorbate causes serious illness like scurvy. Recovery of vitamin C from the renal filtrate by kidneys is most important for ascorbate conservation.

Ascorbate undergoes autoxidation in neutral aqueous solutions, when traces of transition metals are present (Gershoff, 1993), through a two-step process with an intermediate resonance form named ascorbate free radical (AFR). The maintenance of cytosolic reduced ascorbate has been attributed to the dehydroascorbate reductase system (Rose and Bode, 1992), but it is unlikely that the latter enzymes are involved in the maintenance of extracellular ascorbate in its reduced state. That function may be mostly carried out by NADH-AFR oxidoreductase (Minetti *et al.*, 1992; Coassin *et al.*, 1991). Ascorbate autoxidation can be partially prevented in the presence of HL-60 (Alcaín *et al.*, 1991), K562 (Schweinzer *et al.*, 1993), and neuroblastoma (Medina *et al.*, 1992) cells, and yeasts (C. Santos, personal communication). Moreover, intact roots also prevent extracellular ascorbate autoxidation (González-Reyes *et al.*, 1994). The consequence of this prevention may be the availability of ascorbate in its fully reduced state as an oxidative stress protector.

The presence of cells prevents the ascorbate autoxidation in a cell-number-dependent manner (Alcaín *et al.*, 1991), and the features of this prevention appear to be independent of the ascorbate uptake (Schweinzer and Goldenberg, 1992). In fact, short-term stabilization of ascorbate maintains concentrations much higher than those expected by the uptake of ascorbate or dehydroascorbate by leukocytes (Raghoeban *et al.*, 1987). Thus, we proposed the hypothesis that a transplasma membrane redox system could be the enzyme system causing this stabilization by reduction of AFR (Alcaín *et al.*,

[1] Departamento de Biología Celular, Facultad de Ciencias, Universidad de Córdoba, E-14004 Córdoba, Spain.

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1991). In fact, AFR is reduced by NADH in thyroid microsomes (Nakamura and Ohtaki, 1993) and plasma membrane (Villalba *et al.*, 1993b), and induces the oxidation of intracellular NADH in whole cells (Alcaín *et al.*, 1990). However, a variety of factors can affect ascorbate oxidation, and some controversy exists about the enzymatic nature of the extracellular ascorbate stabilization phenomena (Schweinzer *et al.*, 1993).

We will discuss here current results regarding the ascorbate autoxidation in the presence of cells or extracellular ascorbate stablization. This stabilization could be based on ascorbate regeneration from the AFR produced or by stopping ascorbate oxidation due to chelating ions, or both. We are trying to dissociate the enzyme-based part from the chemical effect.

GENERATION OF AFR AS A PROOXIDANT

Ascorbate autoxidation is a two-step process through an intermediate resonance free radical (AFR), leading to the production of dehydroascorbate (DHA), the velocity of the first reaction being higher than that of the second one (Wayner *et al.*, 1986). AFR disappears by disproportionation in a second-order reaction (Bielski *et al.*, 1981). This reaction implies the transfer of one electron between two molecules of AFR and the production of one molecule of ascorbate and one of DHA.

AFR can be produced by several ways. First, by adding equal amounts of ascorbate and dehydroascorbate; second, by addition of ascorbate oxidase to an ascorbate solution, and third, due to traces of transition metals, mainly Cu^{2+}. In each way kinetic equilibrium is reached in which $K = [AFR]_2/([ASC] \times [DHA]) = 10^{-8}$ (Goldenburg *et al.*, 1983), where the constant of equilibrium K is strongly pH-dependent ($pK_1 = 4.1$; $pK_2 = 11.79$).

AFR can act as a prooxidant, being a substrate for reductase activity in many tissues (Scheinder and Standinger, 1965; Iyanagi and Yamazaki (1969); Diliberto *et al.*, 1982). Also, NADH has been shown to be an electron donor for the AFR reductase in plasma membrane (Villalba *et al.*, 1993b; Goldenburg *et al.*, 1983; Morré *et al.*, 1987). AFR also acts as a substrate of the NADH-cytochrome b_5 reductase (Iyanagi and Yamazaki, 1969; Kobayashi *et al.*, 1991).

PROPERTIES OF ASCORBATE STABILIZATION OBSERVED IN THE PRESENCE OF CELLS

Enzyme activities can be characterized by both pH- and temperature-dependent kinetics, but the special nature of the extracellular ascorbate stabilization assay makes assessment by these tests difficult. Ascorbate oxidation is strongly dependent on pH (Buettner, 1988), so neutral pH was maintained in our experiments.

At this pH (7.0), we cannot detect the effect of cells on the ascorbate oxidation when it was mediated by ascorbate oxidase, and only copper-mediated oxidation was prevented by HL-60 cells, in our assays.

We consider that two main aspects differentiate both oxidations. Ascorbate oxidase can act far enough from the cells, so that the free radical produced is inaccessible to the electrons at the cell surface, avoiding its reducing capacity. Moreover, the negative-charged cell glycocalix could also complicate AFR interactions at the plasma membrane (Luft, 1976). On the other hand, AFR produced in the presence of copper could complex with ions, being accessible to the cell surface and thus to the electrons transported there. Copper–ascorbate complex appears to explain the inactivation of lactate dehydrogenase (Nelson *et al.*, 1992).

Copper-mediated ascorbate oxidation is proportional to cupric ion concentration, and its oxidation rates were modified by different cell concentrations in 5-min assays (Fig. 1). In all cases, its efficiency was about 40%, which could represent the total ability of cells to prevent ascorbate oxidation due to the ascorbate regeneration (AFR reduction) by the plasma membrane electron transport, although a chemical

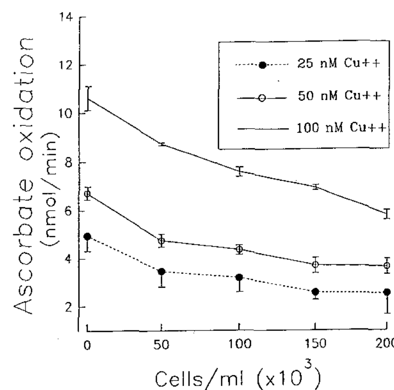

Fig. 1. Ascorbate oxidation versus cell concentration.

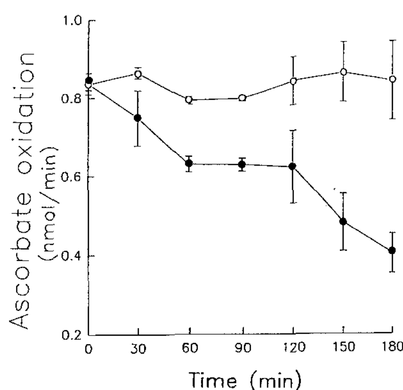

Fig. 2. Ascorbate oxidation versus storage time.

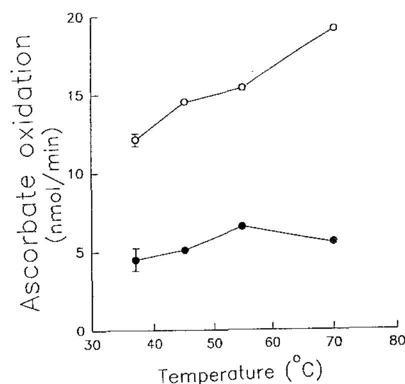

Fig. 3. Ascorbate oxidation versus temperature.

effect of copper chelated at the cell surface could be also considered.

When cells were removed by centrifugation from the incubation media contained in the cuvette, it was not possible to recover the original ascorbate oxidation rates, which were slower than the original. We have not detected protein in these supernatants using Bradford's (1976) method. Thus, these data could not be explained as a simple ion chelation by supernatant proteins, but it is reasonable to consider that significant amounts of copper could be attached to cells. In fact, initial rates of ascorbate oxidation were restored by the extra addition of ion traces. As the difference from the former ascorbate oxidation rate was recovered by removing cells plus the addition of some extra ions, two components are clearly involved, cell activity and ion effects.

Ascorbate stabilization by HL-60 cells was also both time- and temperature-dependent. The long-term storage of these cells in warm buffer (37°C) led to an increase of ascorbate stabilization, which was unchanged in those cells stored at 4°C (Fig. 2). Proteins (0.1 mg/ml) were released from 2×10^5 cells/ml after 4 h incubation in buffer at 37°C, probably due to the death of some cells.

Long-term incubations of K562 cells in buffer led to protein release (Schweinzer *et al.*, 1993). These proteins could explain the long-term ascorbate stabilization in the presence of cells incubated in buffer, apparently by its chelating capacity, although these data neither invalidate the putative enzymatic process of the short-term stabilization of ascorbate caused by intact cells (Alcaín *et al.*, 1991; Medina *et al.*, 1992; Schweinzer and Goldenberg, 1992), nor the ability of cells to stabilize ascorbate when growing in culture media (Alcaín *et al.*, 1990) or in the organism (Minetti *et al.*, 1992).

Ascorbate oxidation rates increased with temperature (Fig. 3), but when cells were present ascorbate oxidation did not significantly change. The capacity of cells to maintain ascorbate stabilization could be due to an increase of its electron flow through the plasma membrane. No protein release was detected in this case.

Cell fixation by glutaraldehyde was also tested for the stabilization of ascorbate. Cells were incubated in 1% glutaraldehyde for 45 min or 2.5% for 15 min, then pelleted and washed twice in buffer (50 mM PBS, pH 7.2). In both cases extracellular ascorbate stabilization by fixed cells was completely abolished (Fig. 4), although the total protein components were present.

Ascorbate is needed in its reduced state for the organisms and there are different mechanisms to maintain it. Thus, for example, AFR is detectable in plasma but not in blood probably because of the reductase activity of blood cells (Minetti *et al.*,

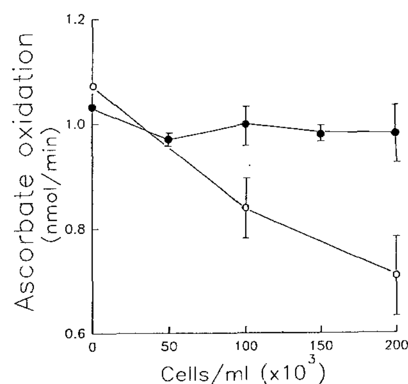

Fig. 4. Ascorbate oxidation versus fixed-cell concentration.

1992). This could be equivalent to the long-term stabilization of ascorbate in whole culture media when cells are present (Alcaín *et al.*, 1990).

When cells are in buffer, long-term incubations lead to protein release, justifying ascorbate stabilization by ions chelation (Schweinzer *et al.*, 1993), although this situation is not comparable to the above conditions.

Further, this chemical effect cannot be applied to the short-term assays for the analysis of ascorbate stabilization. In these cases, no protein release was detected and the activity can be modulated as discussed below.

MODULATION OF THE EXTRACELLULAR ASCORBATE STABILIZATION

Nucleotide Stimulation

Our approach was an attempt to find a physiological regulation of the extracellular ascorbate stabilization using the whole cell. To reach this objective a second messenger modulation was investigated. We found that agents that increase intracellular cAMP, such as forskolin or dibutyryl-cAMP, potentiate the ability of HL-60 cells to keep ascorbate in its reduced state (Rodríguez-Aguilera *et al.*, 1993). Also Rp-cAMP, a membrane-soluble PKA inhibitor, caused the opposite effect. These phenomena taken together with the growth factor (EGF and transferrin) stimulation (Navas *et al.*, 1992), and some sulfonylurea inhibition (unpublished data) of the extracellular ascorbate stabilization by HL-60 cells, give support to the idea of a physiologically modulable system (Alcaín *et al.*, 1991) versus a simple chemical effect (Schweinzer *et al.*, 1993).

AFR, as noted above, has been shown to induce the oxidation of cytosolic NADH (Alcaín *et al.*, 1990). Extracellular addition of lactate, which increases intracellular NADH level, caused a stimulation of the extracellular ascorbate stabilization of HL-60 cells (Rodríguez-Aguilera *et al.*, 1993). Ferricyanide reduction is also stimulated by lactate addition to Ehrlich ascites cells (Medina *et al.*, 1988). This points to a NADH-dependent cAMP-modulable enzymatic ascorbate stabilization similar to the enzyme described for iron reduction in *Saccharomyces cerevisiae* (Lesuisse *et al.*, 1991) and respiratory burst in human neutrophils (Gibson-Berry *et al.*, 1993) except that this reduction depends on NADPH.

Role of Cell Surface

Although no protein has been clearly identified in relation to the extracellular ascorbate stabilization in animal cells, the glycoprotein nature of cell surface has been addressed (Alcaín *et al.*, 1991). Thus, factors affecting the glycocalyx, as well as processes in which the glycocalyx is involved, may affect the extracellular ascorbate stabilization. We have tested the effect of tunicamycin, an agent which interferes with glycosylation (Von Figura *et al.*, 1979), as a mechanism of cell surface glycocalyx alteration. This inhibition partially inhibited the extracellular ascorbate stabilization by HL-60 cells, proportional to tunicamycin concentration used (Rodríguez-Aguilera *et al.*, 1993).

Extracellular ascorbate stabilization by HL-60 cells has been showed to be inhibited by lectins (Alcaín *et al.*, 1991). Also, the NADH-AFR reductase activity is inhibited by these compounds or after digestion with neuraminidase in liver plasma membrane (Navas *et al.*, 1988; Villalba *et al.*, 1993b). Further a glycoprotein has been proposed to be a component of the NADH transdehydrogenase purified from erythrocyte membranes (Wang and Alanpovic, 1978). Also, the redox state of cell surface thiol groups has been reported as an important condition for the NADH-AFR reductase (Villalba *et al.*, 1993a).

Growth State of Cells

The transmembrane redox system has been related with the control of cell growth (see Crane *et al.* (1990) for a review). External AFR has been shown to act as an oxidant and stimulate cell growth (Park and Kimler, 1991; Anderson *et al.*, 1980; Alcaín *et al.*, 1990). On the other hand, exponentially growing Balb/c 3T3 cells showed different extracellular ascorbate stabilization rates than the serum-starved quiescent cells (F. Navarro, personal communication).

Extracellular ascorbate stabilization has also been shown to be hormone-sensitive. Thus, extracellular ascorbate stabilization can be modulated in HL-60 cells and 3T3 cells by several growth factors. This includes EGF and transferrin in HL-60 cells (Navas *et al.*, 1992), and PDGF and ILGF-I in 3T3 cells (Navarro F., unpublished). NADH oxidation by animal cell plasma membrane vesicles has been shown to be stimulated by EGF and diferric transferrin (Morré *et al.*, 1991) and ceruloplasmin (Alcaín *et al.*, 1992). Further, N-myc expression in neuroblastoma

cells also stimulates ascorbate stabilization (Medina *et al.*, 1992).

Quiescency is not the only cellular state that has an influence on the ascorbate stabilization. Differentiation has also been demonstrated to be important for this activity (Rodríguez-Aguilera *et al.*, 1993). HL-60 cell redox activity is modulated during TPA-induced differentiation (Burón *et al.*, 1993), and also increased by Ha-ras expression in mouse embryonic fibroblasts (Crowe *et al.*, 1993). Also, there is a clear modulation of transplasma membrane redox activity between the transformed and nontransformed state of cells (Sun *et al.*, 1986b). Considering the participation of transplasma membrane redox system in the stabilization of extracellular ascorbate, data collected above fit with the general idea of the relationship of plasma membrane electron flow and growth control (Crane *et al.*, 1985).

CONCLUSIONS AND FUTURE PROSPECTS

It is a clear fact that ascorbate is kept in its reduced state in the organisms. The presence of cells prevent ascorbate oxidation leading to its stabilization in buffer. Chelation of transitional ions by proteins and other chelators could justify part of this stabilization, at least for the long-term incubations in buffer. Short-term incubations show features that fit with an enzyme-dependent stabilization, that also can be modulated by growth factors and cAMP, and that has a close relationship with the cell surface components. There is a high similarity with the NADH-AFR reductase, and thus we consider this stabilization, at least most of it, as the regeneration of ascorbate by the reduction of AFR. This hypothesis can only be demonstrated by the purification of the AFR reductase activity components and the reconstitution of the system. Also the binding kinetics of ascorbate and AFR to the cell surface, together with its relationship with copper and iron ions, could give us a clear picture of this property of the cells.

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