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Vitamin C stabilization as a consequence of the plasma membrane redox system

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

Keywords: Ascorbate stabilization; Ascorbate free radical; Plasma membrane redox system; HL-60 cells; Cyclic AMP.

Abbreviations: AFR ascorbate free radicals; FCS fetal calf serum; Sp-cAMPS Sp-cyclic adenosine monophosphothionate; Rp-cAMPS Rp-cyclic adenosine monophosphothionate.

Introduction

Oxygen metabolism generates free radicals which induce hazardous oxidation of biological molecules. Ascorbate (vitamin C) is a water-soluble antioxidant that is found in blood plasma in appreciable concentrations. Ascorbate reacts spontaneously with oxygen and free radicals in a one-electron reaction leading to the production of an intermediate resonance form, ascorbate free radical (AFR). AFR is a relatively non-hazardous radical that decays by disproportionation reaction resulting in dehydroascorbate formation. The maintenance of ascorbate in its reduced state has been related to the dehydroascorbate reductase system (Rose and Bode 1993), although this function may be mostly carried out by NADH-AFR oxidoreductase (Minetti et al. 1992, Coassin et al. 1991).

In vitro ascorbate autoxidation can be partially prevented in the presence of HL-60 (Alcaín et al. 1991), K562 (Schweinzer et al. 1993), neuroblastoma (Medina et al. 1992), or yeast cells (Santos pers. comm.), although it can probably occur in the presence of most kinds of cells. Cells prevent ascorbate autoxidation in a cell-dependent fashion (Alcaín et al. 1991), independently of ascorbic acid uptake (Schweinzer and Goldenberg 1992). The consequence of this prevention is the availability of ascorbate as an oxidative stress protector in the intercellular space. Thus, we propose the hypothesis that transplasma membrane redox system could be the enzyme system causing extracellular vitamin C stabilization (Alcaín et al. 1991). This hypothesis is endorsed by cyclic nucleotide and growth factor modulation of the reaction. Implication of glycoprotein function in this system is also addressed by the data obtained from glycosylation interference experiments.

Material and methods

Culture conditions

HL-60 cells were cultured at 37 °C in RPMI-1640 medium (Sigma, Federal Republic of Germany) supplemented with 10% fetal calf serum (FCS) (Flow, Scotland), 100 units/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml amphotericin B (Sigma), in an humidified atmosphere of 5% CO_2 and 95% air. Cells were concentrated from stock cultures by centrifugation at 1000 **g** for 5 min and washed twice in serum-free RPMI-1640 medium.

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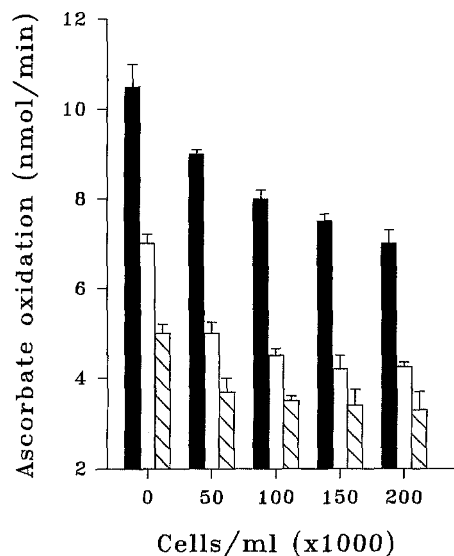

Fig. 1. Short-term ascorbate oxidation rates as a function of the number of cells and copper concentrations: 100 nM (closed bars), 50 nM (open bars) and 25 nM (hatched bars). n = 4

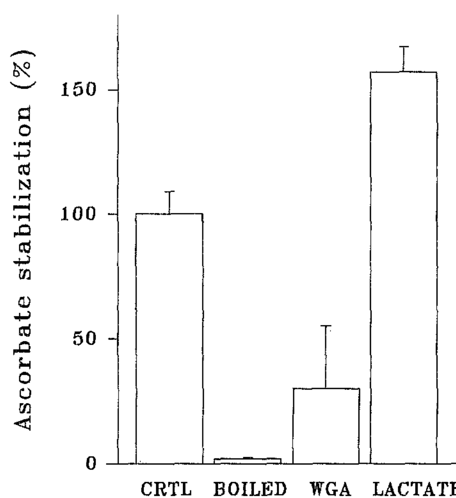

Fig. 2. Effect of WGA, lactate incubation, and cell boiling on the ascorbate stabilization caused by HL-60 cells. n = 3

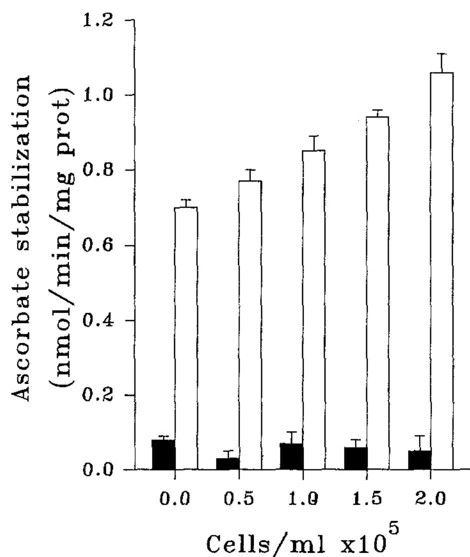

Fig. 3. Effect of glutaraldehyde fixation (closed bars) on the ascorbate stabilization by HL-60 cells. n = 4

Ascorbate oxidation

Short-term ascorbate oxidation was followed by a direct reading at 265 nm for 10 min at 37 °C either in the presence or absence of cells as described (Alcaín et al. 1991). Specific activity of the ascorbate stabilization was calculated by the difference between the rates of ascorbate oxidation with and without cells (Navas et al. 1992). An extinction coefficient of 11.2/mM/cm, determined on the basis of ascorbate absorbance in Tris-HCl buffer, was used in calculations of specific activities. This coefficient is in agreement with previous reports (Wunderling et al. 1986) and allows the measurement of ascorbate with enough sensitivity (Sthal et al. 1985).

Treatments

Incubations with Sp-cAMPS and Rp-cAMPS (Biolog, Federal Republic of Germany) were for 1–3 min in 0.1 M Tris-HCl buffer, pH 7.4, and those with dibutyryl-cAMP and forskolin (Sigma, Federal Republic of Germany) were for 5 min in the growth medium to allow intracellular cAMP accumulation (Yoshikawa et al. 1981). Tunicamycin incubations were for 3 h in the culture medium, conditions leading to a maximal accumulation at the Golgi apparatus (Brown et al. 1984). Lactate (10 mM) incubation was for 30 min in 0.1 M Tris-HCl buffer, pH 7.4. WGA incubation at a final concentration of 300 ng/ml WGA, 1 mM $CaCl_2$, $MnCl_2$, $MgCl_2$, 11 mM glucose in the same buffer. Tunicamycin, lactate or WGA were removed by washing twice in serum-free RPMI-1640 medium. Cells were then collected and resuspended in 0.1 M Tris-HCl buffer, pH 7.4.
Protein was determined using the dye-binding method of Bradford (1976) using bovine τ-globulin as standard.

Results and discussion

Ascorbate chemical oxidation is catalized by copper ions, and is slowed by HL-60 cells in a cell-dependent manner (Fig. 1). The ascorbate oxidation intermediate form, AFR, has been shown to increase the intracellular NAD^+/NADH ratio of HL-60 cells (Burón and Navas 1990). The electron transfer from intracellular NADH to external AFR implies the existence of a transplasma membrane redox system and the reduction of AFR to ascorbate. This correlates with the diminished ascorbate oxidation rate in the presence of cells. The difference between the cell-absent and cell-present assays is presumably the AFR reduced by the plasma membrane redox system using intracellular NADH as electron source.

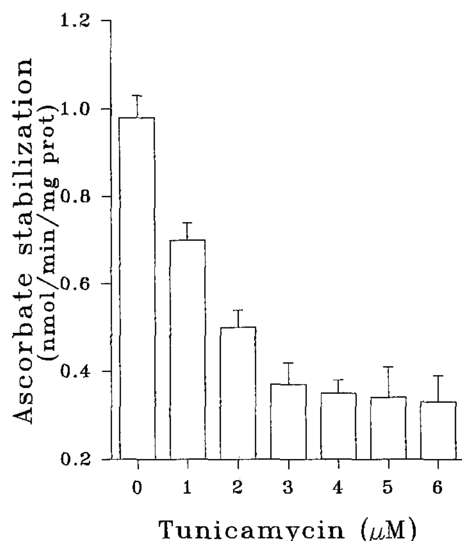

Fig. 4. Extracellular ascorbate stabilization by HL-60 cells as a consequence of tunicamycin incubation. n = 3

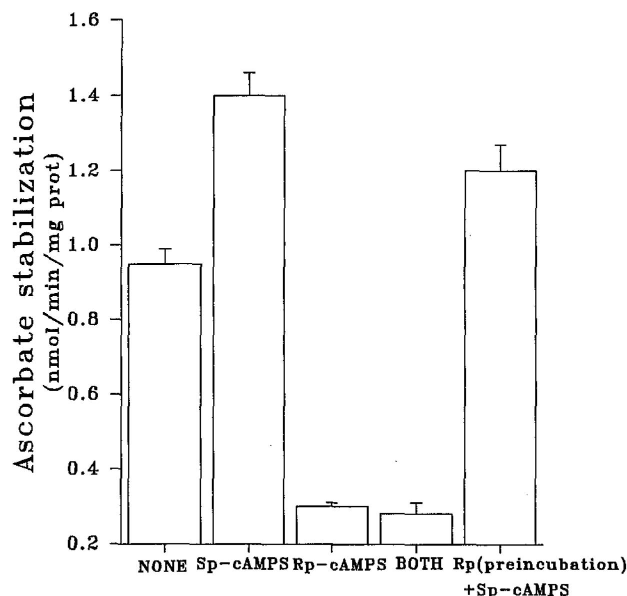

Fig. 5. Role of Sp-cAMPS and Rp-cAMPS on ascorbate stabilization of HL-60 cells. Sp-cAMPS was added at a final concentration of 5 μM for 3 min and Rp-cAMPS final concentration was of 50 μM. n = 3

The enzymatic nature of this activity is addressed since neither boiled (Fig. 2) nor fixed (Fig. 3) cells were able to stabilize ascorbate. Moreover, extracellular addition of lactate to increase cytosolic NADH stimulated the extracellular ascorbate stabilization activity by HL-60 cells (Fig. 2). Further, this activity has been shown to be specifically inhibited by some lectins (Fig. 2). Also, the NADH-AFR reductase of plasma membrane is inhibited by these compounds and glycosidases (Navas et al. 1988, Villalba et al. 1993). Furthermore, to determine whether or not a glycoprotein could be implicated in the ascorbate stabilization activity, tunicamycin, a glycosilation inhibitor agent (Von Figura et al. 1979) was used. Extracellular ascorbate stabilization by HL-60 cells was decreased proportionally to tunicamycin concentrations (Fig. 4), pointing out to some degree of cell surface glycoprotein function.

Most enzymatic processes show some degree of regulation under physiological conditions. Thus, physiological regulation of the extracellular ascorbate stabilization using the whole cell was important for the general acceptance of this test. To reach this objective effects of growth factors and second messengers effects were investigated.

The membrane-permeable cAMP analog, Sp-cAMPS, potentiated the ability of HL-60 cells to stabilize ascorbate. This response was also observed when cAMP-increasing agents, such as forskolin or dibutyril-cAMP, were used (Fig. 5). Rp-cAMPS, a membrane-soluble protein kinase A blocker, caused the opposite effect. Rp-cAMPS inhibited ascorbate stabilization, and Sp-cAMPS incubation did not prevent the Rp-cAMPS-induced effect (Fig. 5). These data suggest a NADH-dependent cAMP-modulable enzymatic ascorbate stabilization, as described for iron reduction in *Saccaromyces cerevisiae* (Lesuisse et al. 1991).

Tunicamycin-treated HL-60 cells, when treated with Sp-cAMPS, showed only a partial stimulation of ascorbate stabilization ability which was only 11% after incubation with 3 μM tunicamycin, compared to the 40% stimulation in control. Whether the cAMP-modulable component is a glycoprotein remains to be elucidated.

Transmembrane redox systems have been shown to be hormone-responsive (Crane et al. 1990). Extracellular ascorbate stabilization has also been shown to be growth factor-sensitive in HL-60 cells, including EGF and transferrin (Navas et al. 1992). *N-myc* oncogene expression seems also to be somehow implicated in the ascorbate stabilization activity of neuroblastoma cells (Medina et al. 1992). Further, ferricyanide reduction is stimulated in *Ha-ras* expressing mouse embryogenic fibroblasts (Crowe et al. 1993).

Long-term incubations of K562 cells in buffer leads to protein release (Schweinzer et al. 1993). These proteins could explain the long-term ascorbate stabil-

ization chelating transition metals. Finally, all the above-mentioned results were obtained in short-term incubations of HL-60 cells in buffer where protein release was not detected. Thus, an enzymatic system for extracellular ascorbate stabilization should be taken into consideration.

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