Volume 334, number 1, 106–108 FEBS 13219 November 1993

No enzymatic activities are necessary for the stabilization of ascorbic acid by K-562 cells

Esther Schweinzer[a], Georg Waeg[b], Hermann Esterbauer[b], Hans Goldenberg[a,*]

[a]*Department of Medical Chemistry, University of Vienna, Waehringerstrasse 10, A-1090 Wien, Austria*
[b]*Department of Biochemistry, University of Graz, Schubertstrasse 1, A-8010 Graz, Austria*

Received 22 September 1993

We disprove that living cells stabilize ascorbate by the activity of a trans plasma membrane semidehydroascorbate reductase. The two processes show different specificities for both substrate and inhibitor. Not only cells but also cell-conditioned buffers stabilize ascorbate as long as compounds with molecular weights above 10 kDa are not removed. The effect is most probably due to chelation of traces of transition metals.

Ascorbic acid; Dehydroascorbic acid; Monodehydroascorbate reductase; K562 cell; Glutathione

1. INTRODUCTION

Ascorbic acid is rapidly oxidized in neutral aqueous solutions through catalytic effects of transition metals [1–3], but it is relatively stable in biological fluids like plasma, serum or urine [4,5]. In blood plasma, ascorbate is protected from oxidation by the complexation of the transition metals, i.e. iron and copper, by transferrin and caeruloplasmin, but also by albumin [3].

However, when traces of free iron or copper are present (iron overload and protein-free or low-protein fluids like vitreous humour or in vitro cell culture media), tissues or cells must prevent the loss of extracellular vitamin C by themselves to avoid severe oxidative stress reactions like lipid peroxidation [6–8]. DHA/ascorbate cycling mechanisms regenerating the reduced from the oxidized form have been described [9–13].

When living cells are added to protein free buffers, ascorbic acid is stabilized. This stabilization has been ascribed to the activity of a monodehydroascorbate reductase, an enzyme located in the plasma membrane which reduces the first oxidation product of ascorbic acid, the ascorbate free radical [14]. The efficiency of the stabilization has even been used as a quantitative measure for the enzyme activity [15]. Such conclusions are overdrawn because the stabilization of ascorbate observed in the presence of cells can be explained in other ways. In this study we clearly distinguish between the monodehydroascorbate reductase activity and the stabilization of ascorbic acid.

*Corresponding author. Fax: (43) (1) 310 7210.

Abbreviations: BSO, buthionine sulfoximine; DHA, dehydroascorbic acid; GSH, reduced glutathione; HPLC, high-pressure liquid chromatography; TBSG, glucose-containing Tris-buffered saline.

2. MATERIALS AND METHODS

DHA was obtained from Fluka (Buchs, Switzerland), BSO from Sigma.

K-562 cells were grown in RPMI 1640 medium containing 2 mM glutamine, 100 U/ml penicillin/streptomycin and 10% fetal calf serum. Logarithmic growth was maintained by two weekly passages.

Glutathione depletion was achieved by overnight incubation with 180 mM buthionine sulfoximine (BSO) [16] and verified by GSH-determination with the enzyme cycling assay [17]. This treatment decreased the GSH-content of the cells to less than 3% (results not shown).

The stability of the generation, respectively, of ascorbic acid was determined in TBSG (140 mM NaCl, 20 mM Tris, 5 mM glucose, pH 7.4) by HPLC with electrochemical detection [18].

Conditioned buffer was obtained by keeping 3 million cells (if not otherwise stated) in 1.5 ml of TBSG for one hour. During this time, no loss of viability could be observed by Trypan blue staining. The cells were then removed by centrifugation.

Low and high molecular mass components of the buffer were separated by centrifugal filtration through a 10 kDa cut-off Millipore Ultrafree-CL membrane.

3. RESULTS AND DISCUSSION

In TBSG 5–50 μM ascorbate was rapidly degraded by autooxidation and subsequent degradation of DHA, as frequently described.

With a starting concentration of 5 μM, ascorbate became undetectable within 4 h (Fig. 1). 50 μM ascorbate completely disappeared within 10 h. This degradation was inhibited by 50 μM desferrioxamine, which confirms the importance of the presence of traces of iron for ascorbate autooxidation. The presence of 3 million K-562 cells slowed down the degradation process.

 Published by Elsevier Science Publishers B.V.

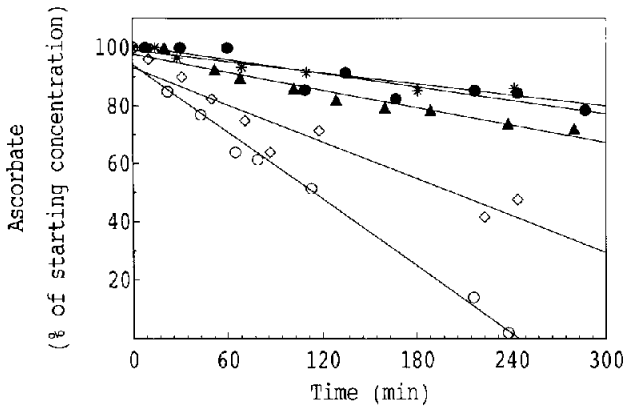

Fig. 1. Disappearance of ascorbate in TBSG-buffer in the absence (○) and in the presence of 50 μM desferrioxamine (◇), 3 million K562 cells (●), cells together with 1 mM thenoyltrifluoro-acetone (△) and glutathione-depleted cells (*), starting from 5 μM ascorbate (100%).

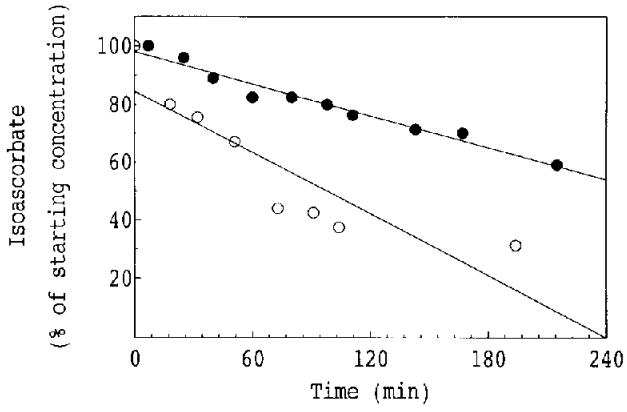

Fig. 2. Stability of isoascorbate in TBSG-buffer in the presence (●) or in the absence (○) of 3 million K562 cells.

1 mM thenoyltrifluoroacetone, an inhibitor of the monodehydroascorbate reductase [19], had no effect on this stabilization. Cells depleted of intracellular glutathione by overnight incubation with 180 μm BSO were as effective as control cells (Fig. 1).

With isoascorbate, the D-isomer of L-ascorbate, a very similar effect was found (Fig. 2). Isoascorbate is not transported into the cells by the ascorbate-specific mechanisms [13] and it is not a substrate for the monodehydroascorbate reductase ([20], Schweinzer, E. and Goldenberg, H., submitted).

When the cells were removed by centrifugation after 1 h and 5 μm ascorbate was added subsequently, it remained stable for a prolonged time. 78% was still present after 5 h and 66% after 15 h (Fig. 3).

One reason for this stabilization could have been complete removal of iron by cellular uptake. In this case addition of fresh TBSG, again containing traces of iron, should lead to enhanced ascorbate degradation. This was not the case. Only addition of 1 μM $FeSO_4$ was able to reinitiate degradation of ascorbate (Fig. 3).

The stabilization of ascorbate was inhibited by 20 μM *p*-hydroxy-mercuribenzoate, a sulfhydryl reagent. This may be taken as evidence for reducing thiols as stabilizing factor(s) (Fig. 3). Two experimental findings argue against this assumption: first, the effect was very long-lasting and there was no sign of exhaustive consumption of putative reducing equivalents (Fig. 3); second, in contrast to the cells themselves, the conditioned buffer was not able to generate ascorbate from DHA or from a mixture of both forms, i.e. a system with a maximal supply of monodehydroascorbate (Fig. 4). As already could be expected from the experiments with the cells, glutathione depletion had no effect on TBSG conditioning (not shown).

To obtain more information on the nature of the stabilizing factor(s), the cell-conditioned buffer was depleted of components with molecular mass higher than 10 kDa by ultrafiltration. This ultrafiltrate was not able to stabilize ascorbate anymore (Fig. 3). The conditioned buffer contained 0.2–1 mg/ml protein. When the cells were homogenized and a similar amount of protein was added to fresh buffer as that found in conditioned buffer after incubation with the cells for one hour, the stabilizing effect could be completely reproduced. 0.6 mg/ml of bovine serum albumin was equally effective (not shown).

The conclusions from these experimental findings are: Monodehydroascorbate reductase activity cannot be assayed as stabilization of ascorbic acid. Both processes show different substrate and inhibitor specificities. Cells can stabilize ascorbic acid by releasing proteins which make transition metals uneffective in catalyzing its autoxidation [1,3,7,8]. This does not exclude the possible function of reducing activities of the cells for monodehydroascorbate or DHA in regenerating ascorbate oxidized by oxygen radicals, oxidized tocopherol or

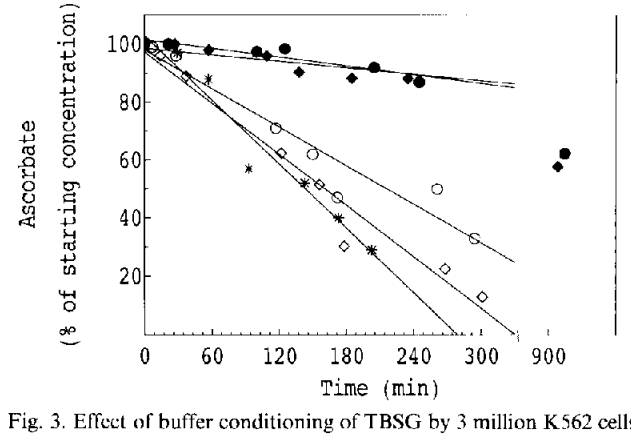

Fig. 3. Effect of buffer conditioning of TBSG by 3 million K562 cells for 1 h on the stability of ascorbate added subsequently (●) and addition of unconditioned fresh buffer (♦), 1 μM $FeSO_4$ (◇) and 20 μM *p*-hydroxybutyrate (○) to it. (*): ascorbate concentration in an ultrafiltrate from conditioned buffer obtained by a 10 kDa cut-off membrane.

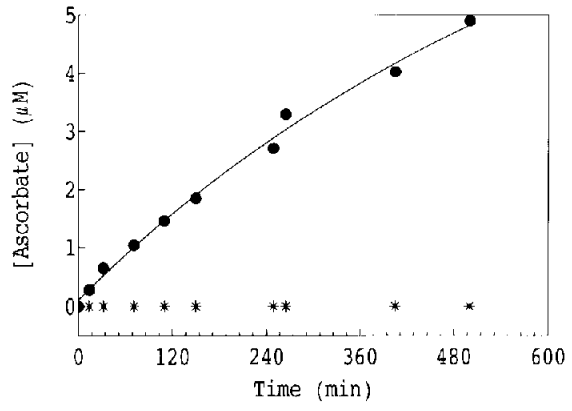

Fig. 4. Ascorbic acid generation from 50 μM DHA in conditioned TBSG alone (*) or in the presence of 3 million cells (●).

other oxidative stress factors. However, experimental setups have to be chosen carefully to discriminate between the three effects.

Acknowledgements: This work was supported by the 'Jubiläumsfonds der Österreichischen Nationalbank', Project 3523.

REFERENCES

[1] Aruoma, O.I. and Halliwell, B. (1987) Biochem. J. 241, 273–278.
[2] Buettner, G.R. (1986) Free Rad. Res. Commun. 1, 349–353.
[3] Gutteridge, J.M.C. and Quinlan, G.J. (1992) Biochim. Biophys. Acta 1159, 248–254.
[4] Dhariwal, K.R., Hartzell, W. and Levine, M. (1991) Am. J. Clin. Nutr. 54, 5384–5387.
[5] Wang, Y.-H., Dhariwal, K.R. and Levine, M. (1992) Ann. N.Y. Acad. Sci. 662, 383–386.
[6] Hershko, C. and Peto, T.E.A. (1987) Br. J. Haematol. 66, 149–151.
[7] Gutteridge, J.M.C., Rowley, D.A., Halliwell, B., Cooper, D.F. and Heeley, D.M. (1988) Clin. Chim. Acta 145, 267 273.
[8] Halliwell, B., Aruoma, O.L., Mufti, G. and Bomford, A. (1988) FEBS Lett. 241, 202 204.
[9] Mann, G.V. and Newton, P. (1975) Ann. N.Y. Acad. Sci. 258, 245–256.
[10] Bigley, R., Wirth, M., Layman, D., Riddle, M. and Stankova, L. (1983) Diabetes 32, 545–548.
[11] Orringer, E.P. and Roer, M.E.S. (1979) J. Clin. Invest. 63, 53–58.
[12] McGown, R.L., Lyons, M.F., Marini, M.A. and Zegna, A. (1990) Biochim. Biophys. Acta 1036, 202–206.
[13] Rose, R.C. (1988) Biochim. Biophys. Acta 947, 335–366.
[14] Alcain, F.J., Buron, I.M., Villalba, J.M. and Navas, P. (1991) Biochim. Biophys. Acta 1073, 380–385.
[15] Navas, P., Alcain, F.J., Rodriguez-Aguilera, J.-C., Villalba, J.M., Morré, D.M. and Morré, D.J. (1992) FEBS Lett. 299, 223–226.
[16] Griffith, O.W. (1982) J. Biol. Chem. 257, 13704–13712.
[17] Anderson, M.E. (1985) Methods Enzymol. 113, 548–555.
[18] Bui-Nguyen, N.H. (1980) J. Chromatogr. 196, 163 165.
[19] Schulze, H.-U., Gallenkamp, H. and Staudinger, Hj. (1970) Biol. Chem. Hoppe-Seyler 351, 809 817.
[20] Schweinzer, E. and Goldenberg, H. (1992) Eur. J. Biochem. 206, 807 812.