Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 1439–1442, March 1985
Cell Biology

Enzyme-controlled scavenging of ascorbyl and 2,6-dimethoxy-semiquinone free radicals in Ehrlich ascites tumor cells

[cytotoxicity/NAD(P)H/cell-surface charge/sulfhydryl groups/electron spin resonance]

RONALD PETHIG, PETER R. C. GASCOYNE, JANE A. MCLAUGHLIN, AND ALBERT SZENT-GYÖRGYI

Laboratory of the National Foundation for Cancer Research, Marine Biological Laboratory, Woods Hole, MA 02543

Contributed by Albert Szent-Györgyi, October 23, 1984

ABSTRACT **The rate of scavenging by Ehrlich ascites cells of anionic ascorbyl and 2,6-dimethoxy-*p*-semiquinone free radicals has been investigated by electron spin resonance spectroscopy both for viable cells and for subcellular fractions obtained by differential centrifugation. The scavenging activity is concluded to be associated with an NAD(P)H enzyme containing an active sulfhydryl group. Attempts to identify the enzyme with the reported properties of either semi-dehydroascorbate reductase or DT-diaphorase have not been successful. The overall free-radical scavenging activity for viable cells is dextrose dependent and is controlled by the coulombic barrier associated with the cell-surface charge. The cytotoxicity of the mixture of ascorbic acid with 2,6-dimethoxy-*p*-benzoquinone is concluded to result from a loss of NAD(P)H reducing power in the cells.**

We have shown (1–3) that a mixture of ascorbic acid and 2,6-dimethoxy-*p*-quinone (2,6-DMQ) can be used to provide a long-lived population of anionic ascorbyl and semiquinone free radicals. The rate at which these radicals are scavenged by Ehrlich ascites cells has been found (2) to be a function of cell viability, of the number of cell-surface sulfhydryl groups, and of the magnitude of the cell-surface charge. Furthermore, in studies (3) of cultured normal and transformed (cancer) cells, the scavenging rates paralleled the state of transformation of these cells. The electrochemical principles involved in the generation of the ascorbyl and semiquinone radicals, as well as their cytotoxicity against ascites tumors in mice, have also been described (1, 2), and the experiments reported here arise from earlier considerations (4) of the oxidation–reduction properties of naturally occurring methoxyquinones.

Further studies are reported here of the dependencies of the free-radical scavenging on cell-surface charge, sulfhydryl groups, and cell viability. The scavenging action of viable cells is dextrose dependent, while that of cellular homogenates requires the presence of NAD(P)H. Studies on ascites cells fractionated by differential centrifugation indicate that the radical scavenging is mediated by a soluble SH-containing enzyme and a dissociable cofactor.

MATERIALS AND METHODS

Ehrlich ascites cells obtained from a cell line maintained in female CD_1 mice (Charles River Breeding Laboratories) were washed in a weakly lysing buffer to eliminate effects associated with the presence of erythrocytes and were then rewashed twice in 0.9% saline.

The technique for generating ascorbyl and 2,6-DMQ radicals in the presence of the ascites cells, as well as the methods used in the electron spin resonance (ESR) measurements of the radical kinetics, have been described (2).

The labile sulfhydryl content of the cells was determined using the Ellman reagent (5), whereby 10^8 ascites cells were suspended in 4 ml of phosphate-buffered saline (pH 7.4) and incubated with Ellman's reagent for 15 min at 22°C. The cells were then centrifuged at 600 × *g* for 10 min, and the spectroscopic absorbance of the supernate was measured at 412 nm. Intracellular glutathione was oxidized using the method of Kosower *et al.* (6), whereby 10^8 ascites cells per ml were incubated for 15 min at 22°C with 0.25 mM diamide (azodicarboxylic acid bis-dimethylamide; Sigma D-3648) and then washed in saline (pH 7.4). For cellular fractionation studies, washed cells were centrifuged at 600 × *g* for 10 min and resuspended in 3 vol of cold 0.25 M sucrose and homogenized using a tight-fitting glass–glass tissue grinder (Thomas) held in ice. The extent of cell destruction was monitored using phase-contrast light microscopy and trypan blue staining. Fractionation in 0.25 M sucrose was carried out using four centrifugation procedures in the following order (average centrifugal forces specified): 600 × *g* for 10 min, 3300 × *g* for 10 min, 12,500 × *g* for 20 min, and 150,000 × *g* for 60 min. According to Londos-Gagliardi *et al.* (7), this procedure yields for ascites cell homogenates the following fractions: a nuclear fraction (pellet P1), a heavy mitochondrial fraction (P2), a light mitochondrial fraction (P3), a microsomal fraction (P4), and a final supernatant S4 containing soluble proteins. Pellets P1, P2, P3, and P4 were twice washed and recentrifuged in 0.25 M sucrose and were finally resuspended in 0.25 M sucrose. Refrigerated centrifuges (Sorvall RC2-B and Beckman L5-65B) were used, and protein was assayed by the method of Lowry *et al.* (8) using bovine serum albumin standards and with the modification that 6 M urea was added to the supernatants and resuspended pellets.

Bovine serum albumin obtained from Sigma and Miles was used for protein standards and, at concentrations of 10 mg/ml, for the ESR studies of supernatant S4. EGTA (Sigma) and EDTA (Sigma) at concentrations of 5 mM were used to test for the possible influence of calcium and multivalent metal ions on the observed radical scavenging activity of the cellular homogenates. The 2,6-DMQ was kindly supplied by G. Fodor (West Virginia University).

RESULTS AND DISCUSSION

The initial studies (2) on the ability of ascites cells to scavenge ascorbyl and semiquinone free radicals were made with the cells suspended in their native ascitic fluid. It was found that when the cells were washed and resuspended in saline (pH 7.4), their radical scavenging ability was significantly diminished even though they appeared to be viable when subjected to trypan blue dye. Scavenging activity could be fully restored by the addition of dextrose at a concentration >0.2 mM. This observation lends support to the earlier conclusion (2) that the radical scavenging activity is dependent

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: 2,6-DMQ, 2,6-dimethoxy-*p*-semiquinone; MalNEt, *N*-ethylmaleimide.

on a cellular metabolic process. As a result of this observation, dextrose at 2 mg/ml (Fisher) was added to all suspensions of washed cells.

In our previous work (2), it was found that the blocking of ascites sulfhydryl groups by *N*-ethylmaleimide (MalNEt) resulted in a decrease of the observed rates of radical scavenging. This effect is shown more fully in Fig. 1, where the scavenging rate per cell is shown to be linearly dependent on the cellular labile sulfhydryl content. The different sulfhydryl levels were obtained by incubation at 22°C with different concentrations of up to 2.0 mM MalNEt (Aldrich) and were assayed using Ellman's reagent (5). As a test of whether intracellular glutathione played a role in the scavenging process, the cells were treated with diamide. This treatment resulted in oxidation of 50% of the intracellular glutathione, as assayed by the method described by Schauenstein *et al.* (9). However, no change was observed in the scavenging ability of the ascites cells, implying that intracellular glutathione is not directly involved.

It was shown (2) that the apparent rate constant k characterizing the scavenging of the monovalent anionic ascorbyl and semiquinone radicals by the ascites cells is given by

$$\ln(k) = \ln(k_0) + F\psi_s/RT. \quad [1]$$

In this expression, ψ_s is the surface potential of the cell membrane and k_0 is the scavenging rate constant that would be observed for an electrically neutral membrane surface. F, R, and T are the Faraday constant, the gas constant, and the absolute temperature, respectively. For low values of ψ_s, it can also be shown (2) that to a good approximation the corresponding membrane surface charge density σ (μC·cm^{-2}) is related to the ionic concentration C (mol·liter^{-1}) of a surrounding bulk aqueous z–z electrolyte by the expression

$$\sigma = 5.89C^{1/2}zF\psi_s/RT. \quad [2]$$

Substitution of Eq. 2 into Eq. 1 gives

$$\ln(k) = \ln(k_0) + 0.170\,\sigma C^{-1/2}z^{-1}, \quad [3]$$

so that a plot of ln (k) versus $C^{-1/2}$ should give a straight line whose slope is proportional to the effective surface charge density of the membrane and inversely proportional to the valency of the z–z electrolyte. As reported earlier (2), increasing the NaCl molarity of an isotonic suspending electrolyte containing ascites cells resulted in an increase of the observed free radical scavenging rate. We have now extended this to include the di-divalent electrolyte $MgSO_4$. As shown in Fig. 2, the straight-line plot of ln (k) versus $C^{-1/2}$ for $MgSO_4$

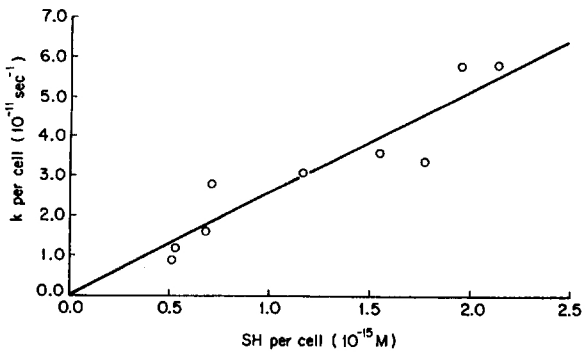

FIG. 1. Variation of the rate constant k characterizing the scavenging of ascorbyl and 2,6-DMQ by ascites cells as a function of labile cellular sulfhydryl content. Details of the determination of the pseudo-first-order rate constant k are provided in ref. 2.

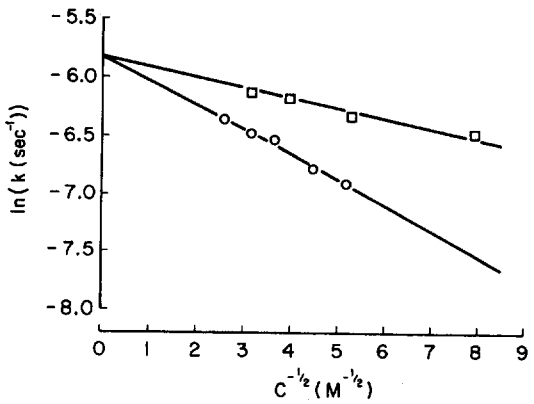

FIG. 2. Variation of the scavenging rate constant k as a function of the reciprocal square root of the electrolyte concentration C for NaCl (○) and $MgSO_4$ (□). The cell-suspending electrolytes were buffered at pH 7.4 with 25 mM Hepes (Sigma) and were kept isotonic to 150 mM NaCl, using sucrose.

has a gradient about one-half that observed for NaCl. The gradient values (−0.089 and −0.207 M$^{1/2}$, respectively) for $MgSO_4$ and NaCl correspond to membrane charge densities of −1.05 and −1.22 μC·cm^{-2}, respectively, which can be compared very favorably to the value of −1.22 μC·cm^{-2} determined previously (2). As discussed elsewhere (2), these surface charge densities are compatible with the electrophoretic mobility results of Weiss (10).

The observation that the free-radical scavenging follows the ion valency relationship predicted by Eq. 3 gives strong support to the concept that the cell-surface charge acts as a coulombic barrier that controls the rate at which the anionic ascorbyl and semiquinone radicals can approach the cell scavenging sites. Although Eq. 2 is based on the approximations inherent in the Gouy–Chapman theory of colloidal electrical double layers, it appears to provide a reasonable description of the electrical potential of ascites cell membranes. Levine *et al.* (11) have discussed refinements of this theory to take into account the more complex charge distributions of cell surfaces.

As a further test of the rate-controlling influence of the cell membrane potential, a known concentration of ascites cells was homogenized, and the radical scavenging properties of the homogenate were investigated. As expected from our previous experience with nonviable cells, no radical scavenging activity was observed for the homogenate described above, and the addition of dextrose resulted in only a very weak scavenging action. However, the addition of 0.25 mM NADH or NADPH to the homogenate resulted in a scavenging rate constant that was 1.9 times that observed for the original concentration of viable cells. Since there was no cell-surface potential to be overcome by the free radicals in the homogenate, the rate constant observed under these conditions can be used as an approximate value for k_0. Eq. 1 then allows the cell-surface potential for the intact cells to be estimated from

$$F\psi_s/RT = \ln(k/k_0) \cong \ln(1/1.9),$$

which yields a surface potential of −16 mV for intact ascites cells in 150 mM NaCl, a value close to that of −14 mV deduced earlier (2) and to the average value of −13 mV derived from Fig. 2 and Eq. 2.

The effect of NAD(P)H in restoring the radical scavenging activity of homogenized ascites cells is shown in Fig. 3. The observed scavenging rates were not influenced by the addition of 5 mM EDTA or EGTA, but were decreased by incu-

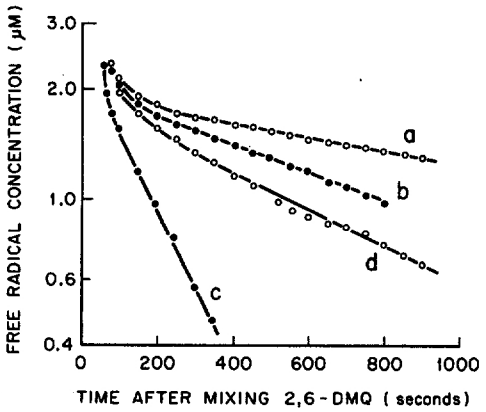

FIG. 3. Decay of the total ascorbyl plus semiquinone free radical concentration for ascites cell homogenate (17.5×10^6 cells per ml). Curves: a, homogenate only; b, homogenate plus dextrose at 2 mg/ml; c, homogenate plus 0.25 mM NADPH; d, homogenate, preincubated with 2.0 mM MalNEt at 22°C for 15 min, plus 0.25 mM NADPH.

bation with MalNEt (Fig. 3) or by preheating above 50°C. These effects indicate that the radical scavenging is associated with a heat denaturable SH-dependent enzyme, and that dissociable calcium and multivalent metal ions are not involved.

The overall time dependency of the free radical population produced in the electrochemical interaction between 2,6-DMQ and ascorbate is the result of the difference between the rate at which ascorbyl and semiquinone radicals are generated and the rate at which they decay. The increase in the decay rate of the free-radical population observed in the presence of ascites cells may arise from the scavenging of free radicals by one-electron enzyme processes. However, it is also possible that such an apparent increase in the radical decay rate is brought about by two-electron enzymic reduction processes that deplete the concentration of unreacted 2,6-DMQ and thereby decrease the free radical generation rate. In viable cells, either or both effects could be operative so that more than one enzyme may be responsible for the observed increase in the radical decay kinetics. A possible candidate for a one-electron transfer enzyme that reacts with free radicals is NADH-semi-dehydroascorbate reductase, which Goldenberg (12) reports to be inhibited by insulin at concentrations between 30 and 60 microunits/ml. However, the addition of 50 microunits of insulin per ml (porcine pancreas; Sigma I-3505) to either viable cells or to cell homogenates had no influence on the observed rates of radical scavenging. It has also been observed (13) that semi-dehydroascorbate reductase activity is calcium dependent; however, ascites cells exposed for 30 min to 5 mM EGTA were found to have the same radical scavenging activity as cells maintained in the presence of 2 mM calcium chloride. A two-electron transfer enzyme possibly responsible for the free radical depletion is DT-diaphorase, an NAD(P)H-dependent enzyme that reduces quinones to their hydroquinones. The strongest known inhibitor (14) of this enzyme is 3,3'-methylene-bis-(4-hydroxycoumarin) (dicumarol), but it was found that the addition of dicumarol (Sigma, M-1390) to cell suspensions or homogenates resulted in no change in the radical scavenging activity.

As shown in Table 1, which summarizes the results obtained for several fractionation experiments on ascites homogenates, the radical scavenging activity was retained in all the supernates S1, S2, and S3. Using the scavenging activity and protein content of supernate S1 as the reference level, then from Table 1 it is seen that the scavenging activity of the various fractions roughly paralleled the distribution of protein. If the scavenging activity and protein content of the washings of pellets P2 and P3 were taken into account, then ≈90% of the original scavenging activity observed in S1 was conserved in S3. Pellet P1, containing nuclei and gross cellular debris, exhibited a low scavenging activity. These results indicate that the major site of the free-radical scavenging was not located within the nucleus or mitochondria.

Table 1. Protein content and radical scavenging activity among various fractions

Fraction	Protein content, %	Scavenging activity, %
S1	100	100
P2	1	1
S2	92	83
P3	5	5
S3	65	77

Distribution of protein content and radical scavenging activity was obtained by differential centrifugation of homogenized Ehrlich ascites cells. Protein content and activity of supernatant S1 were taken as 100%.

Supernate S4, obtained after centrifugation (9×10^6 *g*·min), exhibited the interesting property of possessing a very low radical scavenging activity in the presence of NAD(P)H unless bovine serum albumin (10 mg/ml) (fraction V, Sigma, A-8022) was also added. However, when bovine albumin fraction V (code 81-066) or *S*-cysteinyl bovine albumin (code 81-082), both obtained from Miles, was added to S4, no such restoration of the radical scavenging was observed. These observations imply that the effect of bovine serum albumin was not associated with free sulfhydryl groups or with bovine albumin itself, but with an impurity (or impurities). It is known that bovine albumin can contain trace quantities of other enzymes, hormones, vitamins, and heavy metals. Pellet P4 exhibited an insignificantly small scavenging activity, but when reconstituted into S4, the observed scavenging was similar to that observed for S4 plus bovine serum albumin.

CONCLUSIONS

The scavenging of ascorbyl and 2,6-DMQ free radicals by Ehrlich ascites cells is associated with an enzyme linked to the cellular reductant system NAD(P)H/NAD(P)$^+$. The site of interaction with the radicals appears to involve an active sulfhydryl group, and the overall reaction possibly involves the one-electron reduction of the ascorbyl (semi-dehydroascorbate) and semiquinone radicals to ascorbic acid and hydroquinone. A cofactor, which dissociates from the SH enzyme, appears to be required for the overall reduction of the radicals by NAD(P)H. This cofactor, or an analogue of it, is present as an impurity in bovine serum albumin (fraction V, Sigma, A-8022).

The results of the differential centrifugation experiments have indicated that the dominant free-radical scavenging action of Ehrlich ascites cells is not associated with the nucleus or with mitochondria, and our attempts to identify the active enzyme with reported properties of either semi-dehydroascorbate reductase or DT-diaphorase have not been successful.

The earlier observations (1) concerning the cytotoxicity of the mixture of ascorbic acid with 2,6-DMQ were considered to be associated with the generation of ascorbyl and semiquinone radicals. The work presented here would suggest that the cytotoxicity is associated with the one-electron reduction of these radicals by the cells at the expense of cellular NAD(P)H reducing power. Further support is provided here for the earlier conclusion (2) that the rate of radical

scavenging of the anionic ascorbyl and semiquinone radicals by viable ascites cells is controlled by a coulombic barrier associated with the cell surface charge. The observation (3) that the rate of radical scavenging by transformed Chinese hamster ovary and rat kidney cells is significantly greater than that for their normal counterparts indicates that the techniques reported here can be applied to the study of the differences between normal and cancer cells.

We wish to thank Drs. F. F. Becker, H. Esterbauer, B. Kaminer, C.-N. Lai, and W. W. Wainio for valuable discussions, and Mr. Richard Meany for management of the ascites cell line.

1. Pethig, R., Gascoyne, P. R. C., McLaughlin, J. A. & Szent-Györgyi, A. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 129–132.
2. Pethig, R., Gascoyne, P. R. C., McLaughlin, J. A. & Szent-Györgyi, A. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 2088–2091.
3. Gascoyne, P. R. C., McLaughlin, J. A., Pethig, R., Szent-Györgyi, A., Lai, C.-N. & Becker, F. F. (1984) *Int. J. Quantum Chem. Quantum Biol. Symp.* **11**, 309–314.
4. Szent-Györgyi, A. (1982) *Int. J. Quantum Chem. Quantum Biol. Symp.* **9**, 27–30.
5. Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **82**, 70–77.
6. Kosower, N. S., Kosower, E. M., Wertheim, B. & Correa, W. S. (1969) *Biochem. Biophys. Res. Commun.* **37**, 593–596.
7. Londos-Gagliardi, D., Zenebergh, A. & Aubel-Sadron, G. (1979) *Bull. Soc. Chim. Belg.* **88**, 99–105.
8. Lowry, D. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
9. Schauenstein, E., Esterbauer, H. & Zollner, H. (1977) *Aldehydes in Biological Systems* (Pion, London).
10. Weiss, L. (1965) *J. Cell Biol.* **26**, 735–739.
11. Levine, S., Levine, M., Sharp, K. A. & Brooks, D. E. (1983) *Biophys. J.* **42**, 127–135.
12. Goldenberg, H. (1980) *Biochem. Biophys. Res. Commun.* **94**, 721–726.
13. Sun, I. L., Crane, F. L. & Morre, D. J. (1983) *Biochem. Biophys. Res. Commun.* **115**, 952–957.
14. Lind, C., Hochstein, P. & Ernster, L. (1982) in *Oxidases and Related Redox Systems*, eds. King, T. E., Mason, H. S. & Morrison, M. (Pergamon, Oxford), pp. 321–347.