Interaction of Ascorbate and α-Tocopherol in Resealed Human Erythrocyte Ghosts

TRANSMEMBRANE ELECTRON TRANSFER AND PROTECTION FROM LIPID PEROXIDATION*

(Received for publication, September 11, 1995, and in revised form, February 7, 1996)

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A role for ascorbate-derived electrons in protection against oxidative damage to membrane lipids was investigated in reresealed human erythrocyte ghosts. Incubation of reresealed ghosts with the membrane-impermeant oxidant ferricyanide doubled the ghost membrane concentration of F₂-isoprostan e, a sensitive marker of lipid peroxidation. Incorporation of ascorbate into ghosts during reseling largely prevented F₂-isoprostane formation due to extravesicular ferricyanide. This protection was associated with a rapid transmembrane oxidation of intravesicular ascorbate by extravesicular ferricyanide. Transmembrane electron transfer, which was measured indirectly as ascorbate-dependent ferricyanide reduction, correlated with the content of α-tocopherol in the ghost membrane in several respects. First, ascorbate resealed within ghosts protected against ferricyanide-induced oxidation of endogenous α-tocopherol in the ghost membrane. Second, when exogenous α-tocopherol was incorporated into the ghost membrane during the reseling step, subsequent ferricyanide reduction was enhanced. Last, incubation of intact erythrocytes with soybean phospholipid liposomes, followed by reseling ghost preparation, caused a proportional decrease in both the membrane content of α-tocopherol and in ferricyanide reduction. Incorporation of exogenous α-tocopherol during reseling of ghosts prepared from liposome-treated cells completely restored the ferricyanide-reducing capacity of the ghosts. These results suggest that the transmembrane transfer of ascorbate-derived electrons in erythrocyte ghosts is dependent in part on α-tocopherol and that such transfer may help to protect the erythrocyte membrane against oxidant stress originating outside the cell.

Erythrocytes possess redundant and overlapping mechanisms for protection of their cytoplasmic contents against oxidative damage, including catalase (1), superoxide dismutase (2), and low molecular weight antioxidants such as GSH and ascorbate (3). Defenses against oxidant damage in the plasma membrane are poorly understood but relate primarily to pre- and peroxidation of unsaturated fatty acids in the lipid bilayer (4). Both lipophilic and cytoplasmic factors are thought to protect the erythrocyte membrane against lipid peroxidation. Foremost is α-tocopherol (4, 5), but ubiquinol-10 (6, 7), membrane protein sulfhydryls (8), and a GSH-dependent phospholipid hydroperoxidase (9, 10) are also likely to contribute. Although ascorbic acid does not directly retard peroxidation of membrane lipids (11), it may do so indirectly by reducing the tocopheroxyl free radical in the lipid bilayer (12, 13).

Beyond their ability to use intracellular reducing potential to protect the cell membrane from oxidant stress, erythrocytes have long been known to reduce extracellular oxidants such as ferricyanide (14–16), methemoglobin (17), and ferricytochrome c (18). This export of electrons across the cell membrane has been attributed to activity of a transmembrane NADH:(acceptor) oxidoreductase (E.C. 1.6.99.3) (19, 20). There are probably several such activities in erythrocyte membranes (20). For example, a transmembrane enzyme has been purified (21) that can be differentiated from the prominent inward facing cytochrome b₅ reductase (22) by the presence of carbohydrate, lower substrate affinities, and lack of a flavin as a cofactor. The transmembrane enzyme has also been implicated in the export of electrons derived from ascorbate (15, 16, 23). A physiologic function of this enzyme has not been established. One possibility is that in reticulocytes it provides electrons for reduction of Fe³⁺ on transferrin (24); the resulting Fe²⁺ is then released and can be taken up by the cell (25). It has also been suggested that such an enzyme may use electrons from intracellular NADH to reduce lipid hydroperoxides in the membrane (26). Ascorbate might help to protect the lipid bilayer, either by donating electrons to such an enzyme or by recycling the lipophilic antioxidant α-tocopherol.

The goals of the present study were to evaluate the mechanism of ascorbate-dependent trans- or intramembrane electron transfer in human erythrocytes and to determine whether such transfer protects against membrane lipid peroxidation. To minimize the effects of endogenous cytoplasmic antioxidants and to ensure study of transbilayer transfer, reresealed erythrocyte ghosts containing ascorbate were used. The results show that ascorbate resealed within ghosts protects against ferricyanide-induced loss of endogenous α-tocopherol and against peroxidation of membrane lipids. The protective effect of intravesicular ascorbate involves a transmembrane transfer of ascorbate-derived electrons, the extent of which depends on the membrane content of α-tocopherol.

EXPERIMENTAL PROCEDURES

Materials—Solid L-[1-14C]ascorbic acid (4.7 Ci/mol) and L-[1-3H]glucose (16.2 Ci/mmol were obtained from DuPont NEN. The former was stored in separate aliquots under nitrogen until just before use. Dehydroascorbate (DHA) and tridecylamine were from Aldrich. All other reagents were obtained from Sigma.

Cell Preparation—Human erythrocytes were pre-

*This work was supported by National Institutes of Health Grants DK 26657 and DK 48831, DK 50435, GM 42056, GM 15431, and ES 00267 and by a grant from the Vanderbilt University Research Council. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: DHA, dehydroascorbate; PBS, phosphate-buffered saline; HPLC, high pressure liquid chromatography.
Ascorbate-dependent Electron Transfer in Erythrocyte Ghosts

pared from heparinized blood drawn from normal volunteers. The cells were washed three times in 10 volumes of phosphate-buffered saline (PBS), which consisted of deionized water containing 140 mM NaCl and 12.5 mM Na2HPO4 at pH 7.4. The buffy coat of white cells was carefully removed with each wash. Resteraled impermeable erythrocyte ghosts were prepared from intact cells as described by Stedek and Kant (27).

Measurement of Lipid Peroxidation—Lipid peroxidation was measured by thiobarbituric acid-reactive substances. Thiobarbituric acid-reactive substances were measured as described in the previous section (36).

Measurement of Lipid Peroxidation—For measurement of lipid peroxidation, released ghosts were lysed in 10 volumes of deionized water, and ghost membranes were prepared by three centrifugation washes at 15,000 × g in 10 ml of PBS. F2-isoprostanes were extracted from ghost membranes, purified by TLC, derivatized, separated by selected ion monitoring gas chromatography, and quantified by a gas chromatography/mass spectrometric assay employing stable isotope dilution techniques, as described previously (36). Lipid hydroperoxides in liposomes and erythrocyte ghosts were measured using the FOX2 assay (34). Thioarbituric acid-reactive substances were measured as described previously (37).

Data and Statistical Analyses—Curve fitting was carried out by nonlinear least-squares regression in the graphics software package Figs by SigmaSoft Inc., Cambridge, United Kingdom. Significance was assessed by one-way analysis of variance using the statistical software package Sigmastat (Jandel Scientific, St. Louis, MO).

RESULTS AND DISCUSSION

To induce an oxidant stress on the erythrocyte membrane, released ghosts that had been depleted of cytolsic contents were incubated for 15 min with 1 mM ferricyanide at 37°C.
Ferricyanide has long been known not to penetrate the intact erythrocyte membrane (38). Similarly, the agent also failed to oxidize the residual hemoglobin present in resealed ghosts (results not shown), indicating that any effects observed are restricted to the outer surface of the membrane. However, ferricyanide is a relatively mild oxidant, and it did not increase the ghost membrane content of either thiobarbituric-reactive substances or lipid hydroperoxides (results not shown). Therefore, F2-isoprostanes were measured to provide a more sensitive measure of membrane lipid peroxidation in the ghosts. F2-isoprostanes are derived from free radical-mediated oxidation of arachidonic acid and have been shown to provide specific and extremely sensitive estimates of lipid peroxidation, both in vivo (39) and in vitro (40). In contrast to lipid hydroperoxides, F2-isoprostanes are chemically stable end products that can remain esterified in tissues or be released by the actions of phospholipases (36). Ghosts resealed in the presence or absence of ascorbate had low levels of F2-isoprostanes, and ferricyanide treatment more than doubled F2-isoprostane formation (Table I). In ghosts resealed to contain ascorbate, the membrane content of F2-isoprostanes was halved. These results show that ferricyanide does oxidize a small amount of arachidonic acid that is presumably located in the outer membrane bilayer and that intravesicular ascorbate protects against this oxidation. The findings also indicate the enhanced sensitivity of the F2-isoprostane assay compared with other measures of lipid peroxidation. Although the improved sensitivity of this assay is due in part to the ease of oxidation of arachidonic acid, previous results suggest that this level of oxidation would not be detected by measuring changes in the membrane content of arachidonic acid (41).

Possible mechanisms for ascorbate-dependent protection of the ghost membrane against ferricyanide-induced lipid peroxidation were investigated. It is unlikely that the observed protection was due simply to consumption of most of the oxidant by intravesicular ascorbate, since in these incubations ferricyanide was present in at least a 17-fold molar excess over ascorbate. Whereas ferricyanide apparently does not cross the ghost membrane, ascorbate may efflux from the ghosts and consume ferricyanide directly in the medium. To assess the latter, time-dependent efflux of radiolabel from ghosts that had been loaded with [14C]ascorbate was measured, with the results shown in Fig. 1. In untreated ghosts, the rate of efflux of the carbon-14 label was similar to that observed for L-[3H]glucose, which was used as a marker for nonspecific diffusion. In two additional experiments, after 15 min of incubation at 37 °C, 90% of the carbon-14 radiolabel remaining in the ghosts was ascorbate, as determined by analysis of carbon-14 distribution in the HPLC eluates with correction for zerotime values. On the other hand, only [14C]DHA and 2,3-[14C]diketo-1-gulonic acid were found outside the ghosts at this time; neither ascorbate nor [14C]ascorbate was detected in the buffer. This suggests that the observed efflux of radiolabel from the ghosts occurred as [14C]DHA and not as [14C]ascorbate. Similar conclusions have been reached for intact cells (42–44). However, in intact cells the net efflux of [14C]ascorbate is slower than observed in these ghosts (42, 44). This difference is most likely due to the limited ability of the ghosts to recycle DHA to ascorbate compared with intact cells, so that any DHA generated rapidly exits on the glucose transporter (42, 43).

When ghosts resealed with [14C]ascorbate/L-[3H]glucose were incubated with ferricyanide, there was rapid release of about 70% of the carbon-14, with no effect on the efflux of L-[3H]glucose (Fig. 1). Under similar conditions, 1 mM ferricyanide also decreased the measured intravesicular ascorbate content to less than 5% of control concentrations within 15 min. HPLC analysis showed that following ferricyanide treatment, 90% of the radioactivity inside the ghosts was 2,3-[14C]diketo-1-gulonic acid, and the remainder was [14C]dehydroascorbate and [14C]ascorbate. No [14C]ascorbate was detected outside the ghosts. These results suggest that extravasicular ferriyanide oxidizes intravesicular ascorbate to DHA, which can then either escape the ghost on the glucose transporter (42–44) or undergo irreversible degradation to 2,3-diketo-1-gulonic acid (45). The latter, having undergone ring opening and containing a negative charge, is trapped within the ghosts and accounts for the residual carbon-14 radioactivity observed in the efflux experiment of Fig. 1.

The separation of ferriyanide and ascorbate across the ghost membrane implies a transmembrane transfer of ascorbate-derived electrons, which could be estimated as the extent of ferricyanide reduction outside the ghosts. Ferricyanide reduction occurred rapidly over the same time course as loss of radiolabel from ferriyanide-treated ghosts and was largely complete following 15 min of incubation (results not shown). Several features of this ascorbate-to-ferriyanide electron transfer are shown in Table II. Ghosts resealed to contain ascorbate were from 3–5-fold more effective in reducing extravasicular ferriyanide than ghosts not containing ascorbate.

### Table I

| Agent present during Resealing Incubation | F2-isoprostanes nmol/ml |
|-----------------------------------------|-------------------------|
| None                                    | 1.7 ± 0.9*              |
| None                                    | 4.3 ± 1.2               |
| Ascorbate                               | 1.2 ± 0.4*              |
| Ascorbate                               | 2.0 ± 0.7*              |

**FIG. 1.** Efflux of radiolabel from [14C]ascorbate-loaded resealed erythrocyte ghosts. Erythrocyte ghosts were resealed in the presence of 400 μM unlabeled ascorbate and 0.05 μCi of [14C]ascorbate as well as 0.2 μCi of tracer L-[3H]glucose. Following the usual centrifugation washes, the ghosts were incubated at 37 °C in PBS containing 5 mM d-glucose in the absence (circles) or presence of 1 mM ferricyanide (squares). At the indicated times the efflux of radiolabel from the ghosts was measured as described under "Experimental Procedures." The solid symbols show the efflux of carbon-14, and the open symbols show the efflux of tritium. Data from two experiments are shown ± S.E. Thin solid lines are monoexponential fits to the data, with r > 0.997. The dashed horizontal line shows the residual fraction of radiolabel that would be present in the ghosts at equilibrium.

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When ghosts were incubated with 100 \( \mu \)M DHA, a small but significant increase in ferricyanide reduction was also observed. No increase in intravesicular ascorbate content was detected by direct assay, but after 5 min of incubation with 400 \( \mu \)M \( [14C] \)DHA under the conditions of Table II, 90% of the carbon-14 present in ghosts was \( [14C] \)ascorbate. This suggests that the mechanism for the DHA-induced increase in ferricyanide reduction involved uptake of DHA, intravesicular reduction of DHA to ascorbate, and transmembrane reduction of extravesicular ferricyanide. Although glucose was required for the small DHA effect, omission of glucose did not affect the much larger extent of ferricyanide reduction in ghosts resealed to contain ascorbate.

The addition of ascorbate oxidase to the ghosts several minutes before ferricyanide was added did not affect ferricyanide reduction (Table II). In control studies not shown, the addition of 400 \( \mu \)M ascorbate with ascorbate oxidase under the conditions of Table II decreased the expected amount of ferricyanide reduction by about two-thirds, indicating the ability of the enzyme to compete with ferricyanide for extravesicular ascorbate. The failure to see an ascorbate oxidase-induced decrease in ferricyanide reduction in ghosts containing ascorbate supports the observations shown in Fig. 1, which indicated little direct efflux of ascorbate across the ghost membranes during the experiment. This further suggests a transmembrane transfer of ascorbate-derived electrons in the reduction of ferricyanide, rather than ferricyanide-induced efflux of ascorbate, followed by direct oxidation by ferricyanide outside the ghosts.

Ghosts resealed in the presence of 4 \( \mu \)M GSH showed a doubling in ferricyanide reduction (Table II). GSH and ascorbate together had an additive effect on the extent of ferricyanide reduction. A role for GSH in recycling ascorbate was investigated by measuring intravesicular ascorbate concentrations just before the addition of ferricyanide. No ascorbate was detected in ghosts resealed with GSH alone, but in ghosts resealed with both 400 \( \mu \)M ascorbate and 4 \( \mu \)M GSH, ascorbate depletion was almost completely prevented (data not shown). This suggests that in ghosts resealed with GSH and ascorbate, much of the enhanced ability to reduce ferricyanide was due to increased intravesicular ascorbate concentrations. Direct participation of GSH in the transmembrane electron transfer process cannot be ruled out, however.

A common mechanism was sought to account for both transfer of ascorbate-derived electrons and ascorbate-dependent protection of the outer bilayer leaflet from peroxidation in response to ferricyanide treatment. It has previously been observed that ascorbate alone does not directly transfer reducing equivalents to membrane lipids or hydroperoxides (11). A more likely possibility is that \( \alpha \)-tocopherol might mediate the effect. It is well established that \( \alpha \)-tocopherol can intercept free radicals in lipid bilayers (4) and that ascorbate can recycle \( \alpha \)-tocopherol from its free radical in cell membranes (46, 47). As shown in Fig. 2, increasing amounts of intravesicular ascorbate were found to preserve endogenous \( \alpha \)-tocopherol in resealed ghosts over a range of extravesicular ferricyanide concentrations. This protection was partial at a 400 \( \mu \)M loading concentration of ascorbate and more complete at a loading concentration of 2 \( \mu \)M, with an associated lag phase. The ascorbate-dependent protection of endogenous \( \alpha \)-tocopherol against oxidation by ferricyanide fits with the hypothesis that \( \alpha \)-tocopherol is the proximal electron donor to ferricyanide and that ascorbate in turn recycles \( \alpha \)-tocopherol.

It is also possible that \( \alpha \)-tocopherol contributes to the transmembrane movement of electrons from ascorbate to ferricyanide. It has been shown that \( \alpha \)-tocopherol can directly transfer ascorbate-derived electrons to ferricyanide across artificial liposomal bilayers (48), although transbilayer movement of \( \alpha \)-tocopherol is slow relative to quinones with short hydrophobic tails (49). To determine whether ascorbate-dependent ferricyanide reduction requires \( \alpha \)-tocopherol, two types of experiment were carried out. In the first, \( \alpha \)-tocopherol was added to the ghost membranes during the resealing step. Only about 10% of added \( \alpha \)-tocopherol was recovered in the cell lysate before rescaling of the ghosts (see Table III legend), possibly because of oxidation or failure to stay in solution. However, 7–8% of the total available \( \alpha \)-tocopherol was found in the ghosts, resulting in a 10-fold increase in the \( \alpha \)-tocopherol content (Table III). The presence of exogenous \( \alpha \)-tocopherol in the ghost membranes tripled ferricyanide reduction in ghosts not containing ascorbate and doubled it in ascorbate-containing ghosts (Table III). It should be noted that the incubation time of ghosts with ferricyanide was 5 min in these experiments rather than 15 min as in the experiments shown in Table II and Fig. 2. A shorter incubation time was used to ensure that ascorbate depletion was not limiting for ferricyanide reduction. Although \( \alpha \)-tocopherol intercalates into the membrane bilayer and can
Ascorbate-dependent Electron Transfer in Erythrocyte Ghosts

The Enzymes of Biological Membranes (Martonosi, A. N., ed) pp. 465–510, Plenum Press, New York

Depletion of endogenous α-tocopherol in membranes not treated with ferricyanide (Table III), simple direct oxidation of α-tocopherol could have accounted for only a small portion of the observed increase in ferricyanide reduction.

In the second type of experiment designed to test for a role of α-tocopherol in facilitating the transfer of ascorbate-derived electrons across the ghost membrane, ferricyanide reduction was measured in ghosts depleted of endogenous α-tocopherol. Depletion of endogenous α-tocopherol was accomplished by incubating intact erythrocytes with soybean lecithin liposomes for 2 h followed by centrifugation washes to remove the liposomes. As shown by the first pair of bars in Fig. 3, this treatment decreased both the endogenous α-tocopherol content and the ferricyanide-reducing capacity of subsequently prepared ascorbate-containing resealed ghosts by 40–50%. A similar decrease compared with control was observed when ascorbate was omitted during the resealing step (results not shown).

Whether α-tocopherol was transferred from erythrocytes to the liposomes (51, 52) or oxidized by the liposomes (53) is controversial. Although lipid peroxidation was not detected in either liposomes or ghosts following incubation of intact cells with liposomes in the present studies, the failure to detect quantitatively transfer supports an oxidative loss. If depletion of α-tocopherol by liposome treatment in intact cells is the cause of the decrease in ferricyanide reduction in resealed ghosts, then adding back α-tocopherol during the resealing phase should reverse the effect. As shown by the second pair of bars in Fig. 3, ghosts exposed to α-tocopherol and ascorbate during resealing had the expected increases in measured α-tocopherol content and ferricyanide-reducing capacity (compare to results shown in Table III). The addition of both α-tocopherol and ascorbate during resealing of ghosts derived from liposome-treated cells increased the α-tocopherol content and the capacity to reduce ferricyanide (second pair of bars in Fig. 3). Although the α-tocopherol content of such ghosts did not attain the level observed in α-tocopherol-treated ghosts prepared from cells not exposed to liposomes, the ferricyanide-reducing capacity was fully restored (compare the second and third pairs of bars in Fig. 3). Thus, both α-tocopherol depletion and repletion correlate with ferricyanide reduction in these ghosts, which supports the possibility that endogenous α-tocopherol might play a role in mediating the transmembrane transfer of ascorbate-derived electrons.

Ferricyanide reduction by erythrocytes has usually been attributed to a transmembrane oxidoreductase that uses intracellular NADH as the electron donor (19, 20). Ascorbate has also been implicated as an electron donor for such an enzyme (15, 16), and recent results from this laboratory indicate that ascorbate may be the preferred substrate (23). The present results further suggest that α-tocopherol, either directly or in concert with an enzyme, may also be involved in the electron transfer as well as in protection of membrane lipids against oxidation by external ferricyanide.

TABLE III
Effects of α-tocopherol addition on ferricyanide reduction in resealed ghosts

| Ghost contents | Ferrocyanide | α-Tocopherol | n |
|----------------|-------------|-------------|---|
| None           | 99 ± 11     | 1.6 ± 0.6   | 6 |
| Ascorbate      | 348 ± 34*   | 1.6 ± 0.5   | 6 |
| α-Tocopherol   | 365 ± 55    | 18.8 ± 5.5* | 3 |
| α-Tocopherol + ascorbate | 720 ± 55** | 17.2 ± 4.5* | 3 |

Notes:
- *p < 0.05 compared with controls.
- **p < 0.05 compared with α-tocopherol treatment.

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10582

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