Biochemical and Biophysical Research Communications **267,** 118–123 (2000)
doi:10.1006/bbrc.1999.1906, available online at http://www.idealibrary.com on IDEAL®

Extracellular Reduction of the Ascorbate Free Radical by Human Erythrocytes

James M. May,*,†,[1] Zhi-chao Qu,* and Charles E. Cobb†
**Department of Medicine and †Department of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-6303*

Received November 15, 1999

We investigated the possibility that human erythrocytes can reduce extracellular ascorbate free radical (AFR). When the AFR was generated from ascorbate by ascorbate oxidase, intact cells slowed the loss of extracellular ascorbate, an effect that could not be explained by changes in enzyme activity or by release of ascorbate from the cells. If cells preserve extracellular ascorbate by regenerating it from the AFR, then they should decrease the steady-state concentration of the AFR. This was confirmed directly by electron paramagnetic resonance spectroscopy, in which the steady-state extracellular AFR signal varied inversely with the cell concentration and was a saturable function of the absolute AFR concentration. Treatment of cells *N*-ethylmaleimide (2 mM) impaired their ability both to preserve extracellular ascorbate, and to decrease the extracellular AFR concentration. These results suggest that erythrocytes spare extracellular ascorbate by enhancing recycling of the AFR, which could help to maintain extracellular concentrations of the vitamin.

Ascorbic acid serves as the first line of defense against oxidant stress in plasma (1, 2). Progressive oxidation of ascorbate generates the one- and two-electron oxidized forms of the vitamin, the ascorbate free radical (AFR) and dehydroascorbic acid (DHA), respectively. Unless DHA is recycled back to ascorbate, it can undergo irreversible ring-opening, which results in decreased stores of the vitamin. Although recycling at the AFR stage avoids this risk, it has been studied primarily as an intracellular process (3, 4). Recycling of extracellular AFR is important to consider, since the AFR will be generated in plasma in response to oxidant stress, both directly from scavenging of reactive oxygen species, and as the initial product when ascorbate recycles the α-tocopheroxyl free radical in low density lipoprotein (5).

Evidence that cells can reduce extracellular AFR is inconclusive, however. Neither preservation of extracellular ascorbate by cultured cells, nor facilitation of ascorbate-dependent extracellular ferricyanide reduction by K562 cells (6, 7) provides direct evidence that recycling of the AFR is involved. In this work we addressed this question by measuring the effect of human erythrocytes on the AFR as measured by EPR spectroscopy. The erythrocyte was chosen for study, since in the bloodstream these cells encounter a variety of oxidant stresses, both endogenous from cellular generation of superoxide and H_2O_2 (8), and exogenous in areas of inflammation. Given that the erythrocyte is the most abundant cell in blood, demonstration that it can recycle extracellular ascorbate from the AFR would provide an efficient way to maintain the plasma ascorbate concentration. In this work we present evidence that erythrocytes lower the steady-state extracellular AFR concentration in a manner that is best explained by recycling of the AFR to ascorbate.

EXPERIMENTAL PROCEDURES

Materials. DHA, dehydroascorbic acid; 2,2,6,6-tetramethyl-4-hydroxy-piperidine-*N*-oxyl (Tempol), and tetrapentyl ammonium bromide were from Aldrich Chemical Co. (Milwaukee, WI). Ascorbate oxidase from *Curcubita* species was from Sigma Chemical Co. (St. Louis, MO).

Preparation of human erythrocytes. Human erythrocytes were prepared from freshly drawn heparinized blood from normal volunteers. The cells were washed three times in ten volumes of phosphate-buffered saline (PBS), which consisted of deionized water containing 140 mM NaCl and 12.5 mM Na_2PO_4, pH 7.4. The buffy coat of white cells was removed with each wash.

Assay of ascorbate oxidase activity. Ascorbate oxidase activity was measured spectrophotometrically. Incubations contained varying initial concentrations of ascorbate (25–500 μM) and 0.2 units/ml ascorbate oxidase in 50 mM Tris-HCl buffer, pH 7.4. The decrease in

Abbreviations used: AFR, ascorbate free radical; DHA, dehydroascorbic acid; PBS, phosphate-buffered saline; Tempol, 2,2,6,6-tetramethyl-4-hydroxy-piperidine-*N*-oxyl.

[1] To whom correspondence should be addressed at 715 Medical Research Building II, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-6303. Fax: 615-936-1667. E-mail: james.may@mcmail.vanderbilt.edu.

0006-291X/00 $35.00

absorption at 265 nm was followed over 1–4 min at 37°C following addition of the oxidase. The rate of ascorbate disappearance was calculated based on an extinction coefficient of 3.3 mM^{-1} cm^{-1} for ascorbate, corrected for a blank sample that did not contain the enzyme.

Ascorbate and GSH measurements. Intracellular ascorbate was measured following cell lysis and ultrafiltration by high performance liquid chromatography with electrochemical detection. The assay was identical to that previously described (9), except that tetrapentyl ammonium bromide was used as the ion pair reagent. Medium ascorbate concentrations were measured after cell removal as follows. An aliquot of the medium was diluted with 4 volumes of ice-cold methanol, allowed to sit on ice for 5 min, microfuged, and taken for assay of ascorbate. Where noted, endogenous erythrocyte ascorbate was depleted by three successive incubations with 1 mM Tempol (9). GSH was measured using the method of Hissen and Hilf (10).

Measurement of ascorbate-dependent ferricyanide reduction. Washed erythrocytes at a 5% hematocrit were incubated at 37°C in PBS that contained 5 mM D-glucose, the indicated concentration of ascorbate (1–20 μM), and either no ascorbate oxidase or 2 U/ml oxidase. After 10 min of incubation, 1 mM potassium ferricyanide was added and incubations were continued for another 30 min. Pre-incubation with ascorbate oxidase was used to generate DHA that would be taken up by the cells and rapidly converted to ascorbate (11). Parallel incubations were also carried out with ascorbate and ascorbate oxidase alone in the absence of cells. At the completion of the incubation, samples containing cells were microfuged to pellet the cells. Duplicate aliquots of the cell supernatant as well as buffer from incubations without cells were diluted 10-fold for determination of ferrocyanide. Ferrocyanide was measured as previously described for human erythrocytes (12) by the method of Avron and Shavit (13), using 1,10-phenanthroline as indicator. Ferricyanide reduction by added ascorbate in the presence of cells was compared to the sum of direct reduction of ferricyanide by ascorbate in the absence of cells and cell-dependent ferricyanide reduction due to intracellular ascorbate, either endogenous or accumulated as a result of uptake and reduction of DHA generated by the oxidase.

Measurement of the AFR by EPR spectroscopy. Time-dependent changes in the AFR concentration were measured by EPR spectroscopy. EPR data were acquired on a Bruker EMX 8/27 spectrometer equipped with a BVT300 variable temperature controller. Sample temperature was maintained at 37°C during data acquisition by blowing precooled nitrogen into the cavity through the front optical port. X-band EPR spectra were collected using a ER041XG-DHA microwave bridge and ER4103TM/9614 cavity. Sample incubations were carried out in a Wilmad WG 804 aqueous flow flat cell. Based on a preliminary incubation, the magnetic field was fixed at value that corresponded to the maximum signal intensity of the low field resonance line of the AFR (arrow, inset spectrum in Fig. 4). The conversion time of the spectrometer was chosen to result in a sweep time of 167 seconds (since each data set contained 2048 points, the timing resolution was approximately 0.082 seconds/point). Other spectrometer settings were: 0.5 G modulation amplitude, 100 kHz modulation frequency, and 10 mW microwave power. The flat cell was pre-loaded with either buffer or a suspension of erythrocytes at the indicated hematocrit to permit tuning of the spectrometer. The experiment was initiated by starting the time-sweep to record a baseline signal. After about 10 sec, the indicated concentration of ascorbate oxidase was added to the remainder of the pre-warmed sample, which was rapidly injected into the flat cell. Approximately 15 sec elapsed between addition of ascorbate oxidase and the actual start of data recording on the mixed sample. The concentration of the AFR was determined by calibration of the instrument using the signal of 10 μM Tempol.

Data analysis. Data are shown as mean ± SE from the indicated number of experiments. Curve-fitting was performed with the graphics analysis program Origin (Microcal Software, Inc., Northhampton, MA).

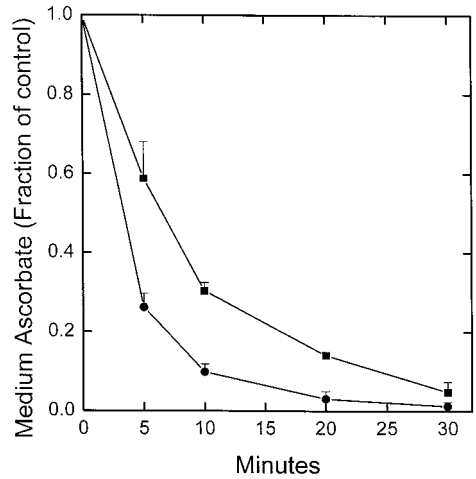

FIG. 1. Preservation of extracellular ascorbate by erythrocytes. Ascorbate oxidase was added with mixing to a final concentration of 20 mU/ml of total volume to PBS at 37°C that contained 50 μM ascorbate, 5 mM D-glucose, and either no cells (circles) or 40% erythrocytes (squares). At the indicated times, aliquots of each incubation were removed, microfuged for 30 s, and the supernatant was diluted with 5 volumes of ice-cold methanol to stop the reaction. Ascorbate concentrations are shown from 3 experiments as a fraction of the initial value, which was 38 ± 4 and 45 ± 6 μM in the absence and presence of cells, respectively.

RESULTS

If erythrocytes can recycle the AFR to ascorbate, then addition of cells should decrease the rate of ascorbate disappearance caused by ascorbate oxidase. As shown in Fig. 1, this was the observed effect, since 40% cells significantly slowed the loss of extracellular ascorbate. Assuming the sparing of ascorbate was due to its regeneration, the cells were able to regenerate ascorbate at a rate of 1.8 μM/min over the initial 5 min of incubation. Addition of the cells raises the extracellular concentrations of both ascorbate and its oxidase compared to incubations without cells. For this reason, results are shown as a fraction of the initial value. Possible explanations other than recycling of extracellular ascorbate were considered. First, a measured 15–20% increase in the extracellular ascorbate concentration due to the presence of cells could have saturated the oxidase. The apparent K_m of ascorbate oxidase under these conditions in the absence of cells was found to be 88 ± 6 μM (N = 6), so that an increase in the initial extracellular ascorbate concentration to 45 μM due to the cells would not saturate the enzyme. Second, incubation with cells could have inhibited the oxidase. However, ascorbate oxidase activity was unaffected by incubation of the enzyme with either intact cells or ghosts derived from cells under the conditions of Fig. 1 (results not shown). Finally, the cells could

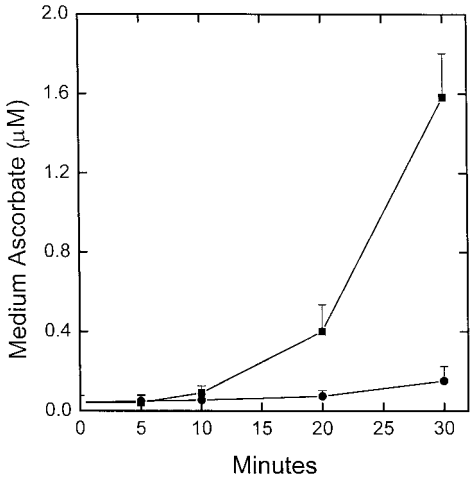

FIG. 2. Efflux of ascorbate from erythrocytes. Erythrocytes at a 40% hematocrit were incubated in the absence (circles) or presence (squares) of 50 μM DHA in PBS that contained 5 mM D-glucose for 10 min at 37°C, washed by centrifugation three times in 5 volumes of PBS, suspended to a 40% hematocrit, and incubated with mixing in glucose-containing PBS at 37°C. At the indicated times, aliquots of cells and buffer were removed, the cells were pelleted for 30 s in a microfuge, and portions of the clear supernatants were taken for assay of ascorbate. Data are shown from 3 experiments.

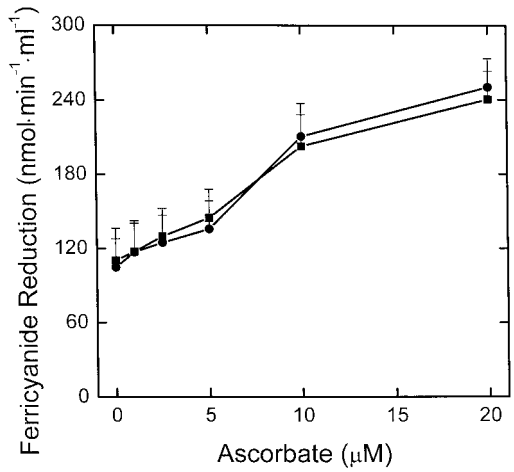

FIG. 3. Reduction of ferricyanide by extracellular ascorbate. Incubations and measurements of ferricyanide reduction were carried out as described under Experimental Procedures. Ferricyanide reduction in the presence of cells (circles) is compared to the sum of direct ferricyanide reduction by ascorbate plus ferricyanide reduction as a result of uptake and reduction of the same concentration of DHA generated by ascorbate oxidase (squares). Data are shown from 4 experiments and expressed per ml of erythrocyte cytoplasm, which was taken as 70% of the cell volume (20).

have released endogenous ascorbate into the medium. As shown in Fig. 2, such release was negligible from basal cells, and low from cells that had been loaded with 50 μM DHA. A nine-fold increase in intracellular ascorbate (from 41 $\pm$ 7 to 362 $\pm$ 83 μM in control and DHA-treated cells, respectively) resulted in only 1.6 $\pm$ 0.2 μM ascorbate in the medium after a 30 min incubation. Additional experiments were carried out to determine whether this preservation of extracellular ascorbate by erythrocytes is due to reduction of the AFR at the cell surface.

Schweinzer and Goldenberg (6, 7) used the cell-impermeant oxidant ferricyanide both to oxidize extracellular ascorbate to the AFR, and to provide an integrated measure of the ability of cells to recycle the AFR. We also found that incubation of erythrocytes with low but increasing extracellular concentrations of ascorbate caused a progressive increase in the rate of extracellular ferricyanide reduction (Fig. 3). However, this increase was entirely accounted for by the sum of 1) direct ferricyanide reduction by added ascorbate, and 2) by an increase in trans-membrane ferricyanide reduction due to uptake and reduction of DHA by the cells. Therefore, at least in erythrocytes, reduction of ferricyanide cannot be used to measure extracellular AFR reduction.

More direct evidence that erythrocytes can reduce extracellular AFR was sought in EPR studies. The AFR was generated in this system by incubation of 1 mM ascorbate with 50 mU/ml ascorbate oxidase. The amplitude of the low-field line of the resulting AFR signal (Fig. 4, inset) was then followed for 2 min. Higher ascorbate concentrations were used than in the studies shown in Fig. 1 to ensure that there was adequate steady-state AFR signal, and that the fall in the ascorbate concentration during the incubation was relatively small (i.e., the ascorbate concentration remained well above the K_m of the oxidase). This resulted in stable AFR signals during the incubations (Fig. 4). In the absence of added ascorbate, the oxidase did not generate an AFR signal in the presence of cells (results

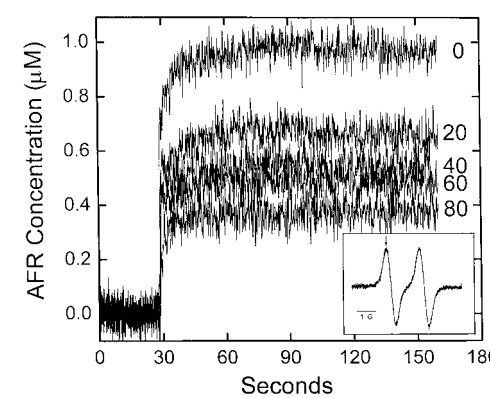

FIG. 4. Effects of increasing cell concentrations on extracellular AFR measured by EPR. Erythrocytes at the indicated concentrations were added to PBS at 37°C that contained 5 mM D-glucose, 1 mM ascorbate, and 50 mU/ml ascorbate oxidase. EPR spectra were obtained as described in Experimental Procedures. The inset shows the AFR signal, with an arrow indicating the location on the low-field line that was followed as a function of time. Percent packed cell volumes are indicated on the right side of the figure. Results from one of three experiments performed are shown.

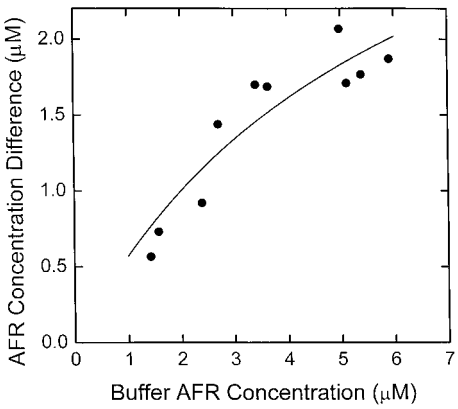

FIG. 5. Effects of the AFR concentration on cell-dependent decreases in the extracellular AFR. Erythrocytes at an 80% hematocrit were incubated at 37°C in PBS that contained an initial concentration of 1 mM ascorbate, 5 mM D-glucose, and ascorbate oxidase concentrations varying from 10 to 200 mU/ml. The steady-state AFR concentration measured in the presence of cells was subtracted from that measured in the absence of cells to derive the cell-dependent decrease in the steady-state AFR concentration. Results are shown from 2 experiments fit to an hyperbolic plot with kinetic parameters as noted in the text.

not shown). Thus, the AFR signal produced when cells were incubated with ascorbate oxidase and ascorbate must have been due to oxidation of extracellular ascorbate. Incubation with increasing concentrations of cells caused progressive decreases in the steady-state AFR signal compared to incubation without cells (Fig. 4). These results should not be affected by an increase in concentrations of the ascorbate and the oxidase due to an increase in the volume occupied by the cells, since the AFR concentration is assessed over the entire volume of the flat cell and since the oxidase was already saturated with ascorbate.

The difference in AFR signal generated by ascorbate oxidase in the presence and absence of 80% cells was considered to reflect the cell-dependent AFR reductase activity. This activity increased in a saturable manner in response to increases in the measured AFR concentration, as shown in Fig. 5. The apparent K_m of this effect, determined over the range of AFR concentrations studied, was 6 ± 3 μM, with a maximal difference of 4 ± 1 μM.

Pretreatment of erythrocytes with a 2 mM concentration of the thiol reagent *N*-ethylmaleimide irreversibly inhibited both the ability of the cells to preserve extracellular ascorbate (Fig. 6A), and their ability to decrease the AFR signal in the EPR experiment (Fig. 6B). The inhibition was partial for both measurements, averaging 22% in the ascorbate preservation experiments, and 27% in the EPR experiments. In studies not shown, intracellular GSH was completely alkylated by concentrations of *N*-ethylmaleimide over 1 mM under these conditions. The effect of *N*-ethylmaleimide was apparent when both 40% (Fig. 6A) and 80% erythrocytes (Fig. 6B) were studied. The poorly penetrant thiol reagent *p*-chloromercuribenzene sulfonic acid at a concentration of 0.2 mM was without effect in the ascorbate preservation experiments, and had inconsistent effects in the EPR experiment.

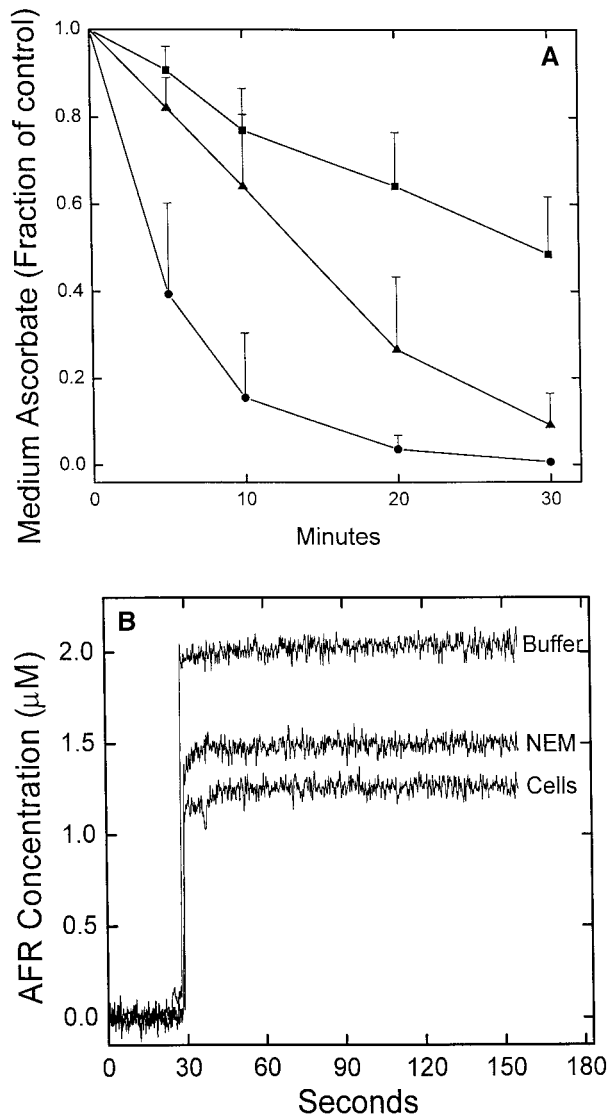

FIG. 6. Effects of *N*-ethylmaleimide pretreatment on the ability of erythrocytes to preserve extracellular ascorbate and to decrease the extracellular AFR. Cells at a 40% hematocrit were incubated at 37°C in PBS that contained 5 mM D-glucose in the presence or absence of 2 mM *N*-ethylmaleimide. After 15 min, the cells were washed 3 times by centrifugation in PBS and suspended to a 40% hematocrit for the ascorbate preservation experiment as described in the legend to Fig. 1 (A: no cells, circles; cells, squares; cells + *N*-ethylmaleimide, triangles, N = 3 experiments). In the studies shown in A, initial concentrations of extracellular ascorbate were: no cells, 55 ± 7 μM; cells, 65 ± 12 μM; and cells + *N*-ethylmaleimide, 64 ± 12 μM. In the EPR experiment (representative of 3 such experiments performed) shown in B, cells were suspended to an 80% hematocrit, and conditions were the same as those described in the legend to Fig. 4.

DISCUSSION

This work provides evidence that human erythrocytes can recycle extracellular AFR to ascorbate. This conclusion derives from the findings that during extracellular oxidation of ascorbate to the AFR by ascorbate oxidase, the cells preserve extracellular ascorbate (Figs. 1 and 6), but decrease steady-state extracellular AFR concentrations (Fig. 4). This is the expected effect if the cells reduce the AFR to back to ascorbate. For reasons noted under Results, it cannot be explained by cell-induced changes in the kinetics of the ascorbate/ascorbate oxidase system used to generate the AFR. The observed preservation of ascorbate also makes it unlikely that the cell-dependent decrease in the AFR concentration is due to oxidation of the AFR to DHA, or to uptake and intracellular reduction of the AFR. Regarding the latter, at physiologic pH, the AFR carries a negative charge (14) and is thus no more likely than ascorbate to enter the cells. Ascorbate uptake (12, 15), like its efflux (Fig. 2), is very slow in erythrocytes. Despite the uncertainties necessitated by the use of intact cells, these results strongly suggest that erythrocytes reduce extracellular AFR to ascorbate.

Previous evidence for a cell-surface AFR reductase activity was indirect in that it was not based on measurements of the AFR. Nucleated cells have been shown to preserve extracellular ascorbate in culture (16, 17), and this was taken as evidence for a cell-surface AFR reductase (18). However, as demonstrated by Schweinzer *et al*. (19), ascorbate preservation could be due to factors released from the cells, especially during long term incubations. Cell-dependent reduction of ferricyanide by low micromolar concentrations of extracellular ascorbate has also been considered to reflect activity of a cell-surface AFR reductase in K562 erythroleukemic cells (6, 7). However, at least in erythrocytes, this effect was entirely accounted for by the sum of direct reduction of ferricyanide by added ascorbate plus trans-membrane reduction of ferricyanide by increases in intracellular ascorbate (Fig. 3). The latter is due to uptake and reduction of DHA that resulted from extracellular ascorbate oxidation by ferricyanide. The trans-membrane reduction of ferricyanide has been attributed to a trans-plasma membrane oxidoreductase activity that uses intracellular ascorbate as its primary electron donor (12, 20, 21). In the studies of Schweinzer and Goldenberg (6, 7), DHA-enhanced ferricyanide reduction was also measured separately, and accounted for most (~80%) of the ascorbate effect. Although ferricyanide could, in principle, be used to measure the activity of a cell-surface AFR reductase, it reacts even faster with the AFR than with ascorbate (22), and removes the substrate for the cell-surface reductase.

The mechanism of trans-membrane electron transfer to extracellular AFR is unknown. Since it was unaffected by *p*-chloromercuribenzene sulfonic acid, it does not appear to involve cell surface sulfhydryls or the trans-membrane ferricyanide reductase, which is sensitive to inhibition of exofacial sulfhydryls by this agent (23, 24). Treatment of erythrocytes with *N*-ethylmaleimide at concentrations sufficient to alkylate essentially all intracellular GSH impaired both the ability of the cells to preserve ascorbate (Fig. 6A) and their ability to suppress the AFR generated by ascorbate and ascorbate oxidase (Fig. 6B). This could indicate reaction with sensitive sulfhydryl or amino groups on a trans-membrane protein, or inhibition of intracellular GSH metabolism and a decrease in electrons available for trans-membrane export. The source of intracellular electrons for extracellular AFR reduction is unknown. Intracellular AFR reductases usually use NADH for this purpose (3, 25), although trans-membrane AFR reduction in chromaffin granules by cytochrome b_{561} uses ascorbate as the donor (26, 27).

A thiol-sensitive ubiquinone-dependent AFR reductase activity has been described in plasma membranes from liver (28, 29) and K562 cells (18). However, this activity, which has been demonstrated only in open membranes, involves cytochrome b_5 reductase (29, 30). The latter is well known to reduce the AFR using NADH as an electron donor (31), but is exposed only on the cytoplasmic membrane face. Since ubiquinone is considered to be restricted to the interior of the lipid bilayer (32), and poorly accessible to hydrophilic reducing agents such as ascorbate (33, 34), it is difficult to see how this complex could form a trans-membrane AFR reductase without other components. Our data do not distinguish between a trans-membrane protein and a transfer complex involving lipid-soluble electron transfer agents such as ubiquinone or α-tocopherol.

The ability of erythrocytes to reduce the AFR to ascorbate may be of physiologic importance, given the abundance of the erythrocyte and its proximity to oxidant stress generated in the vascular bed. The latter might occur in areas of atherosclerosis, ischemia, or inflammation (5, 35). The measured AFR concentration in normal human plasma, even following oxidation and stabilization by dimethylsulfoxide, was only about 70 nM (36). In areas of oxidant stress, the AFR will increase, but should remain in the effective range of the erythrocyte reductase activity, which has an apparent K_m of about 6 μM (Fig. 5). The ability of erythrocytes to rapidly recycle ascorbate from the AFR stage is quantitatively more important than release of ascorbate from the cells. Under similar incubation conditions, even ascorbate-loaded cells released ascorbate at only 0.05 μM/min (Fig. 2), compared to a rate of 1.8 μM/min for ascorbate regeneration by cells (Fig. 1). Whether ascorbate preservation by erythrocytes is due to a trans-membrane enzyme or some other effect of the cells, the present results suggest that the effect occurs at the AFR stage, and that it may be an important

mechanism for ascorbate regeneration in the bloodstream.

ACKNOWLEDGMENT

This work was supported National Institutes of Health Grant DK 50435.

REFERENCES

1. Frei, B., Stocker, R., and Ames, B. N. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 9748–9752.
2. Frei, B., England, L., and Ames, B. N. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 6377–6381.
3. Lumper, L., Schneider, W., and Staudinger, H. (1967) *Hoppe Seylers. Z. Physiol. Chem.* **348,** 323–328.
4. Diliberto, E. J., Jr., Dean, G., Carter, C., and Allen, P. L. (1982) *J. Neurochem.* **39,** 563–568.
5. Diaz, M. N., Frei, B., Vita, J. A., and Keaney, J. F., Jr. (1997) *N. Engl. J. Med.* **337,** 408–416.
6. Schweinzer, E., and Goldenberg, H. (1992) *Eur. J. Biochem.* **206,** 807–812.
7. Schweinzer, E., and Goldenberg, H. (1993) *Eur. J. Biochem.* **218,** 1057–1062.
8. Van Dyke, B. R., and Saltman, P. (1996) *Free Radical Biol. Med.* **20,** 985–989.
9. May, J. M., Qu, Z.-C., and Mendiratta, S. (1998) *Arch. Biochem. Biophys.* **349,** 281–289.
10. Hissin, P. J., and Hilf, R. (1976) *Anal. Biochem.* **74,** 214–226.
11. May, J. M., Qu, Z. C., Whitesell, R. R., and Cobb, C. E. (1996) *Free Radical Biol. Med.* **20,** 543–551.
12. May, J. M., Qu, Z.-C., and Whitesell, R. R. (1995) *Biochemistry* **34,** 12721–12728.
13. Avron, M., and Shavit, N. (1963) *Anal. Biochem.* **6,** 549–554.
14. Laroff, G. P., Fessenden, R. W., and Schuler, R. H. (1972) *J. Am. Chem. Soc.* **94,** 9062–9073.
15. Okamura, M. (1979) *J. Nutr. Sci. Vitaminol. (Tokyo)* **25,** 269–279.
16. Alcain, F. J., Buron, M. I., Villalba, J. M., and Navas, P. (1991) *Biochim. Biophys. Acta* **1073,** 380–385.
17. Minetti, M., Forte, T., Soriani, M., Quaresima, V., Menditto, A., and Ferrari, M. (1992) *Biochem. J.* **282,** 459–465.
18. Gomez-Diaz, C., Rodriguez-Aguilera, J. C., Barroso, M. P., Villalba, J. M., Navarro, F., Crane, F. L., and Navas, P. (1997) *J. Bioenerg. Biomembr.* **29,** 251–257.
19. Schweinzer, E., Waeg, G., Esterbauer, H., and Goldenberg, H. (1993) *FEBS Lett.* **334,** 106–108.
20. Orringer, E. P., and Roer, M. E. (1979) *J. Clin. Invest.* **63,** 53–58.
21. May, J. M., Qu, Z.-C., and Whitesell, R. R. (1995) *Biochim. Biophys. Acta* **1238,** 127–136.
22. Iyanagi, T., Yamazaki, I., and Anan, K. F. (1985) *Biochim. Biophys. Acta* **806,** 255–261.
23. Cherry, J. M., Mackellar, W., Morré, D. J., Crane, F. L., Jacobsen, L. B., and Schirrmacher, V. (1981) *Biochim. Biophys. Acta* **634,** 11–18.
24. Schipfer, W., Neophytou, B., Trobisch, R., Groiss, O., and Goldenberg, H. (1985) *Int. J. Biochem.* **17,** 819–823.
25. Schulze, H.-U., Gallenkamp, H., and Staudinger, H. (1970) *Hoppe Seylers. Z. Physiol. Chem.* **351,** 809–817.
26. Njus, D., Knoth, J., Cook, C., and Kelley, P. M. (1983) *J. Biol. Chem.* **258,** 27–30.
27. Wakefield, L. M., Cass, A. E. G., and Radda, G. K. (1986) *J. Biol. Chem.* **261,** 9739–9745.
28. Villalba, J. M., Canalejo, A., Burón, M. I., Córdoba, F., and Navas, P. (1993) *Biochem. Biophys. Res. Commun.* **192,** 707–713.
29. Villalba, J. M., Navarro, F., Córdoba, F., Serrano, A., Arroyo, A., Crane, F. L., and Navas, P. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 4887–4891.
30. Navarro, F., Villalba, J. M., Crane, F. L., MacKellar, W. C., and Navas, P. (1995) *Biochem. Biophys. Res. Commun.* **212,** 138–143.
31. Ohnishi, T., Yamazaki, H., Iyanagi, T., Nakamura, T., and Yamazaki, I. (1969) *Biochim. Biophys. Acta* **172,** 357–369.
32. Ulrich, E. L., Girvin, M. E., Cramer, W. A., and Markley, J. L. (1985) *Biochemistry* **24,** 2501–2508.
33. Hauska, G. A. (1977) *FEBS Lett.* **79,** 345–347.
34. Ilani, A., and Krakover, T. (1987) *Biophys. J.* **51,** 161–167.
35. Ross, R. (1999) *N. Engl. J. Med.* **340,** 115–126.
36. Pietri, S., Seguin, J. R., d'Arbigny, P. D., and Culcasi, M. (1994) *Free Radical Biol. Med.* **16,** 523–528.