CHAPTER 4

Erythrocytes reduce extracellular ascorbate free radicals using intracellular ascorbate as an electron donor

This chapter was adapted from MM VanDuijn, K Tijssen, J VanSteveninck, PJA Van den Broek, and J Van der Zee, Erythrocytes reduce extracellular ascorbate free radicals using intracellular ascorbate as an electron donor. J Biol Chem, 275(36): 27720-27725, 2000

Summary

Ascorbate is readily oxidized in aqueous solution by ascorbate oxidase. Ascorbate radicals are formed, which disproportionate to ascorbate and dehydroascorbic acid. Addition of erythrocytes with increasing intracellular ascorbate concentrations decreased the oxidation of ascorbate in a concentration-dependent manner. Concurrently, it was found, utilizing ESR spectroscopy, that extracellular ascorbate radical levels were decreased. Control experiments showed that these results could not be explained by leakage of ascorbate from the cells, inactivation of ascorbate oxidase, or oxygen depletion. Thus, this means that intracellular ascorbate is directly responsible for the decreased oxidation of extracellular ascorbate. Exposure of ascorbate-loaded erythrocytes to higher levels of extracellular ascorbate radicals, resulted in the detection of intracellular ascorbate radicals. Moreover, efflux of dehydroascorbic acid was observed under these conditions. These data confirm the view that intracellular ascorbate donates electrons to extracellular AFR via a plasma membrane redox system. Such a redox system enables the cells to effectively counteract oxidative processes and thereby prevent depletion of extracellular ascorbate.

Introduction

Ascorbate, a well-known anti-oxidant in biological systems, is capable of reducing a variety of oxidative compounds, especially free radicals (1, 2). The importance of sufficient levels of ascorbate has been well established in the past (3, 4). Primates and guinea pigs lack the ability to synthesize ascorbate from glucose and are dependent on dietary intake. Ascorbate deficiency may lead to scurvy and to oxidative injury resulting in necrosis or apoptosis. It may also cause malignant proliferation of cells as a consequence of oxidative DNA damage (5).

The oxidation of ascorbate is a two-step reaction in which single electrons are transferred. The first step yields a relatively stable radical, the ascorbate free radical (AFR). In the second step, AFR donates a second electron, yielding dehydroascorbic acid (DHA). These steps are reversible, but DHA can irreversibly be hydrolyzed to diketo-gulonic acid, which degrades further to potentially toxic compounds (6-8). The degradation of DHA is very fast, with a half-life of approximately 8 min at 37 °C (9, 10). Under oxidative stress, the consumption of ascorbate can be high, and without regeneration, ascorbate would soon be depleted. However, in order to restore ascorbate levels, systems exist that reduce AFR and/or DHA. Most of these systems are located in the cell. Reactions proceed spontaneously, such as glutathione-mediated ascorbate regeneration (11), or are

mediated by enzymes, such as thioredoxin reductase (12-14), glutaredoxin (15), and proteindisulfide isomerase (16). Moreover, an NADH:AFR reductase has been described that reduces intracellular AFR (17).

These pathways only convert oxidized ascorbate species that are present in the cell. Regeneration of extracellular ascorbate is more complicated. Reduction of extracellular DHA can involve transport of DHA into the cell, e.g., by the GLUT-1 glucose transporter (9, 18-20). This regeneration pathway, however, cannot prevent depletion of extracellular ascorbate. Ascorbate is trapped inside the cell after reduction and will only slowly leak back to the extracellular space. It is, therefore, essential that other regenerating pathways exist. Recently, Himmelreich et al. reported that, in erythrocytes, extracellular DHA can be reduced without entering the cell (21). Also, transmembrane AFR-reductases have been described in the plasma membrane of liver cells (22) and red blood cells (23, 24). It was suggested that the latter reduce extracellular ascorbate radicals using intracellular NADH, thereby generating ascorbate and NAD^+.

Previous observations indicated that alternative systems might exist in the plasma membranes of cells. It was found that intracellular ascorbate acted as electron donor for transmembrane electron transport, with ferricyanide as extracellular electron acceptor (9, 25-27). However, the actual substrate is still unknown, as ferricyanide is not a physiological compound. Because ascorbate is a natural compound in, for example, blood plasma, we hypothesized that under physiological conditions, AFR and/or DHA might act as the extracellular electron acceptor. Therefore, we investigated this hypothesis using erythrocytes as a model system. The data in this chapter provide the first experimental evidence for the presence of a system in the erythrocyte membrane that reduces ascorbate free radicals in the extracellular space using intracellular ascorbate as an electron donor.

Experimental Procedures

Chemicals were obtained from Sigma or Baker, unless indicated otherwise. L-Ascorbate oxidase (EC 1.10.3.3) was purchased as sticks containing 17 U of enzyme (Roche Diagnostics, Almere, The Netherlands) and was dissolved in phosphate-buffered saline (PBS). Tris(ethylenediamine)-nickel(II) chloride 2-hydrate ($Ni(en)_3^{2+}$) was synthesized according to State (28).

Erythrocytes were obtained from 1-day old citrate-anticoagulated blood, collected from healthy human volunteers by the Bloodbank Leiden/Haaglanden. The cells

were washed three times with PBS. The buffy coat of white cells was removed carefully with each wash.

Erythrocytes were loaded with ascorbate by resuspension of the cells to a hematocrit of 20% in PBS containing 2.5 mM adenosine as energy source (29) and DHA at concentrations as indicated under 'Results'. Control erythrocytes were treated similarly, but without DHA present. After 30 min of incubation at room temperature, erythrocytes were washed three times with PBS, and used within 1 h for subsequent experiments. All experiments were performed in PBS at room temperature.

Ascorbate concentrations in erythrocytes were determined by HPLC. Loaded, packed erythrocytes were mixed with three volumes of 7 mM potassium phosphate, 1 mM EDTA, pH 4.0, and frozen in liquid nitrogen. After thawing, hemoglobin was removed using an Amicon Micropartition System with 30 kD cut-off ultrafiltration membranes (Millipore, Etten-Leur, The Netherlands). 100 µl 0.5 mM EDTA, 450 µl methanol and 5 µl concentrated HCl were added to 200 µl of ultrafiltrate, and 100 µl of this mixture was injected on an Adsorbosphere SAX column (250 x 4.6 mm, Alltech, Breda, The Netherlands). Ascorbate was eluted with 7 mM potassium phosphate, 7 mM KCl, pH 4.0 at a rate of 1.5 ml/min. The LKB 2140 detector (LKB Bromma, Sweden) was set at 265 nm, and chromatograms were integrated on a personal computer, using Nelson 2600 revision 4.1 software (Nelson Analytical, Cupertina, CA, USA). After the elution of ascorbate, the column was regenerated with 0.25 M potassium phosphate and 0.5 M KCl, pH 5.0. Ascorbate concentrations are expressed relative to the water content of packed erythrocytes, which is 70% of the packed cell volume (26).

Ascorbate concentrations were determined by measuring the absorption at 265 nm in a Beckman DU-65 spectrophotometer (ε=14,500 M^{-1} cm^{-1}) or by HPLC analysis. For HPLC analysis, 200 µl of the solution was diluted with 100 µl of 0.5 mM EDTA, 450 µl of methanol and 5 µl of concentrated HCl, and subsequently injected and analyzed as described above. When present, erythrocytes were removed by centrifugation prior to the determination of ascorbate.

Ascorbate depletion from erythrocytes was carried out by treating cells with 1 mM 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (TEMPOL), as described by May *et al.* and Mehlhorn (30, 31). Briefly, 20% erythrocytes were incubated in PBS with 1 mM TEMPOL for 5 minutes at 37 °C. After subsequent centrifugation, this treatment was repeated twice, followed by three washes with PBS alone.

NADH levels in erythrocytes were modulated by incubation of 10% erythrocytes with 10 mM pyruvate, xylitol or glucose at 37 °C for 45 min. After centrifugation,

samples were taken for the NAD/NADH assay. The remaining erythrocytes were resuspended in their respective buffers including pyruvate, glucose or xylitol, and immediately used for an ascorbate oxidation assay. NAD^+ and NADH levels were determined using an alcohol dehydrogenase cycling assay modified from Wagner and Scott (32). Briefly, 200 µl packed erythrocytes were diluted in 1.3 ml extraction buffer, frozen in liquid nitrogen, thawed, and filtered using an Amicon Micropartition System with 30 kD cut-off ultrafiltration membranes. The filtrate was further analyzed as described by Wagner and Scott (32).

ESR spectra were recorded on a JEOL-RE2X spectrometer operating at 9.36 GHz with a 100 kHz modulation frequency and equipped with a TM_{110} cavity. Samples were transferred to a quartz flat cell using a rapid sampling device, which allowed the recording of spectra to be started within seconds after mixing. ESR spectrometer settings were as follows: microwave power, 40 mW; modulation amplitude, 1 G; time constant, 0.3 s; scan time, 5 min; scan width, 15 G.

Loading of erythrocytes with L-[carboxyl-^{14}C]ascorbate (Amersham International) was carried out by incubating a 20% suspension with 500 µM ^{14}C-labeled ascorbate and 1.7 U/ml ascorbate oxidase. After 30 min, the erythrocytes were washed three times with PBS, and resuspended at 10% hematocrit. After the experiment, the suspensions were centrifuged, and the supernatants were transferred to vials for liquid scintillation counting and for immediate HPLC analysis, as described above. Fractions were collected and analyzed by liquid scintillation counting. The scintillation cocktails used were Emulsifier Scintillator 299 and Flo-Scint IV (Packard).

^{14}C-Labeled DHA was prepared by incubation of [^{14}C]ascorbate with bromine, according to Washko et al., with every step on ice (33). Immediately after preparation, 1 mM ^{14}C-labeled DHA was added to either PBS or to a 10% suspension of ascorbate-loaded or control erythrocytes. After incubation, supernatants were collected, separated by HPLC and analyzed by liquid scintillation counting.

Oxygen consumption was measured using a YSI 5300 Biological Oxygen Monitor (YSI Inc., Yellow Springs, OH).

All experiments were performed at least three times and were evaluated using Student's t-test where applicable.

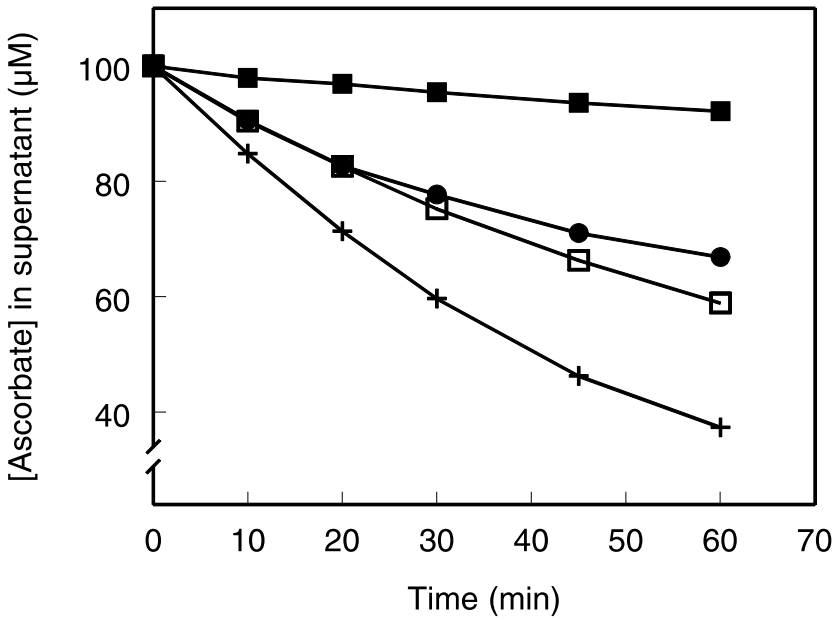

Figure 1. Oxidation of ascorbate by ascorbate oxidase. Ascorbate (100 µM) and 4 mU/ml ascorbate oxidase were incubated at room temperature in PBS (+), supplemented with 10% erythrocytes (●), 10% ascorbate-loaded erythrocytes (■) or 10% ascorbate-loaded erythrocytes treated with TEMPOL (□). At the indicated times, samples were taken, centrifuged and the ascorbate concentration in the supernatant was determined by measuring A_{265}. Ascorbate-loading was performed by incubating cells with 500 µM DHA as described under 'Experimental Procedures'. Results are expressed as the average of three experiments. S.E. values fall within the markers.

Additions	Ascorbate oxidation rate	NADH
	µM/min	% reduced nucleotide
None	1.30 ± 0.05	26 ± 7
Adenosine	1.06 ± 0.09	78 ± 5
Glucose	1.08 ± 0.10	52 ± 9
Xylitol	1.14 ± 0.04	58 ± 9
Pyruvate	1.39 ± 0.06	19 ± 5
TEMPOL	1.39 ± 0.06	14 ± 5

Table 1. Effect of energy sources and TEMPOL on NADH levels and the ascorbate oxidation rate. Erythrocytes that had not been loaded with ascorbate were preincubated with the additions listed. Subsequently, NADH levels and oxidation rates of extracellular ascorbate were determined. All treatments and assays were performed as described under 'Experimental Procedures'. NADH levels are expressed as a percentage of total nucleotides, i.e. NAD^+ + NADH (48 nmol/ml packed cells). All results are shown as an average ± S.E.

Results

Oxidation of ascorbate by ascorbate oxidase

Ascorbate can easily be oxidized in aqueous solution by spontaneous, metal-ion or enzyme-catalyzed reactions. In this study, the enzyme ascorbate oxidase was used to oxidize ascorbate. Incubation of ascorbate with ascorbate oxidase resulted in a decrease of the ascorbate concentration that was linear for 15 min (Fig. 1). After 60 min, approximately 60% of the ascorbate was oxidized. Ascorbate oxidation decreased upon addition of erythrocytes, and decreased even further by addition of erythrocytes, loaded with ascorbate. The addition of cells with high levels of intracellular ascorbate resulted in complete protection (Fig. 1). This suggested that intracellular ascorbate played an important role in the effect of erythrocytes on the oxidation of ascorbate. To test this, ascorbate-loaded cells were treated with TEMPOL, which depleted intracellular ascorbate for more than 90% (data not shown). As expected, the effect of ascorbate-loaded cells on ascorbate oxidation could be reversed by treatment with TEMPOL (Fig. 1). In subsequent experiments, erythrocytes were pre-incubated with various concentrations of DHA to determine the correlation between the intracellular ascorbate concentration and extracellular ascorbate oxidation. Figure 2 shows that the rate of ascorbate oxidation in the external medium was strongly dependent on the intracellular ascorbate concentration: with increasing intracellular ascorbate concentrations the oxidation of extracellular ascorbate decreased.

Figure 1 shows that addition of control erythrocytes (i.e. not loaded with ascorbate) decreased ascorbate oxidation with 30-40%. To test whether NADH contributed to this effect, erythrocytes were incubated with various compounds that are known to perturb the NADH/NAD^+ ratio (34, 35). A decrease in the intracellular NADH concentration resulted in an increase in the ascorbate oxidation rate, whereas an increase in NADH resulted in a concomitant decrease in the ascorbate oxidation rate (Table 1). This shows that NADH, as well as ascorbate, plays a role in the regeneration of extracellular ascorbate. To measure the relative contribution of endogenous ascorbate to the effect of control erythrocytes on ascorbate oxidation, cells were treated with TEMPOL. However, it was found that TEMPOL also reduced intracellular NADH levels (Table 1). It was, therefore, not possible to separate the effects of endogenous ascorbate and NADH. It should be noted that TEMPOL depleted NADH only in the case of control erythrocytes, and not in erythrocytes loaded with ascorbate.

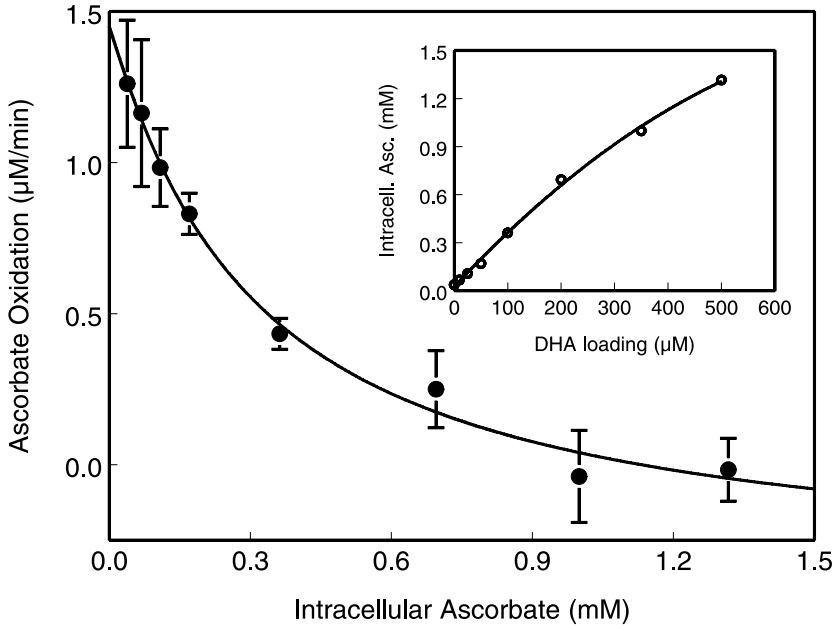

Figure 2. Correlation between the intracellular ascorbate concentration and the oxidation rate of extracellular ascorbate. Erythrocytes were loaded with ascorbate by treating cells with various concentrations of DHA, as described under 'Experimental Procedures'. Subsequently, a 10% suspension was incubated with 100 µM ascorbate and 4 mU/ml ascorbate oxidase. After 0 and 15 min, the amount of ascorbate in the supernatant was determined by measuring A_{265} and by HPLC, and the rate of ascorbate oxidation was calculated. Results of A_{265} measurements are presented as the average of three experiments ± S.E. Measurements using the HPLC method are not shown, but produced similar data. The intracellular ascorbate concentration was determined as described under 'Experimental Procedures'. Inset: Intracellular ascorbate concentration resulting from preincubation with various concentrations of DHA.

The effect of erythrocytes on ascorbate oxidation could be explained by inhibition of ascorbate oxidase by the erythrocytes. To study this hypothesis, control or ascorbate-loaded erythrocytes were incubated with ascorbate oxidase for 0 or 15 min. After removal of the erythrocytes, 100 µM ascorbate was added to the supernatants, and the rate of ascorbate oxidation was determined. It was found that incubation of ascorbate oxidase with control or ascorbate-loaded erythrocytes did not affect the activity of the enzyme. Oxygen is also required for ascorbate oxidase activity. Under our experimental conditions, oxygen levels did not decrease more than 20%, which showed that oxygen levels were not limiting during the experiments (data not shown).

A possible explanation for the protection by ascorbate-loaded erythrocytes could be leakage of ascorbate from the erythrocytes. More than 1 mM of ascorbate was accumulated intracellularly after incubation with DHA (Fig. 2, inset). Leakage of

Extracellular additions	Leakage
	% of total content
None	1.1 ± 0.3
100 µM ascorbate	1.3 ± 0.1
100 µM DHA	1.5 ± 0.1
4 mU/ml ascorbate oxidase	1.2 ± 0.2
14 mU/ml ascorbate oxidase	0.8 ± 0.1
100 µM ascorbate, 4 mU/ml ascorbate oxidase	3.0 ± 0.5
100 µM ascorbate, 14 mU/ml ascorbate oxidase	12.4 ± 1.2
100 µM ascorbate, 4 mU/ml ascorbate oxidase, 20 µM cytochalasin B	1.8 ± 0.3
100 µM ascorbate, 14 mU/ml ascorbate oxidase, 20 µM cytochalasin B	4.2 ± 0.4

Table 2. Leakage of radioactivity from erythrocytes containing [^{14}C]ascorbate. Erythrocytes were loaded with ^{14}C-labeled ascorbate, as under 'Experimental Procedures', washed, and incubated as a 10% suspension with the additions indicated in the table. Leakage after 15 min was determined by centrifugation of the suspensions. Radioactivity in the supernatant was determined by liquid scintillation counting. Values are shown as mean ± S.E. (n=8), relative to the amount of ^{14}C that would be released by lysis of all cells.

the accumulated ascorbate could result in an extracellular ascorbate concentration of up to 100 µM. To investigate leakage of intracellular ascorbate, erythrocytes were loaded with ^{14}C-labeled ascorbate. Table 2 shows that incubation of these erythrocytes caused some leakage of ^{14}C-labeled material, both in control cells and after addition of ascorbate, DHA or ascorbate oxidase. The addition of 100 µM ascorbate and 4 mU/ml ascorbate oxidase resulted in a small increase in leakage of radioactivity. This amounted to an extracellular concentration of 3 µM ^{14}C-labeled material. From the data in figure 2, it can be derived that complete protection against oxidation of ascorbate would need leakage of 20 µM of ascorbate. This means that efflux of ascorbate could maximally account for 15% of the observed effect. Only in the presence of a higher ascorbate oxidase concentration did leakage become substantial. Furthermore, HPLC analysis revealed that most of the radioactivity was released from the cells as [^{14}C]DHA, and not as [^{14}C]ascorbate (Fig. 3). Leakage of DHA could be inhibited by the addition of cytochalasin B, an inhibitor of the GLUT-1 transporter (Table 2). Thus, it can be concluded that leakage of ascorbate is not responsible for the decrease in ascorbate oxidation.

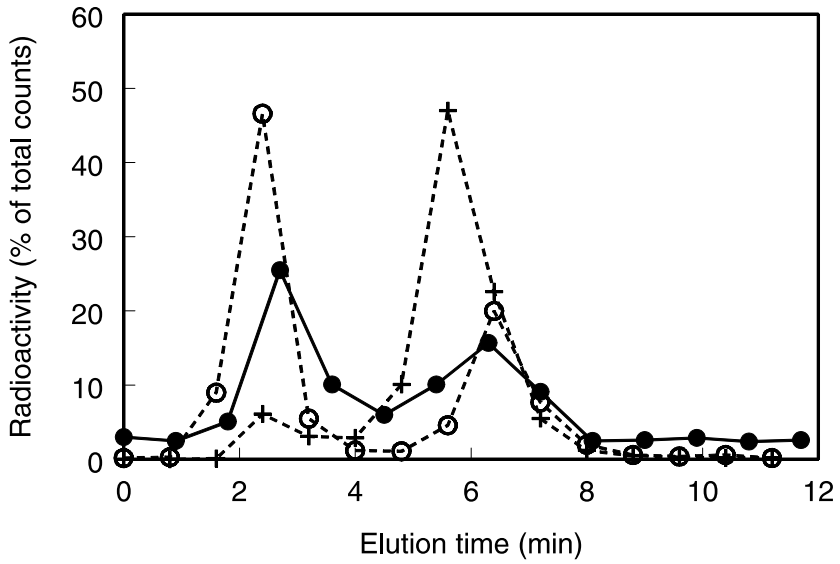

Figure 3. HPLC analysis of radioactive material released from erythrocytes loaded with [^{14}C]ascorbate and exposed to ascorbate and ascorbate oxidase. Erythrocytes were loaded with [^{14}C]ascorbate, as described under "Experimental Procedures". Subsequently, a 10% suspension was incubated with 100 µM ascorbate and 4 mU/ml ascorbate oxidase. After 15 min, the suspension was centrifuged, and the supernatant prepared for HPLC analysis, as described under 'Experimental Procedures'. Counts from the supernatant (●) are shown and compared with the peaks of [^{14}C]ascorbate (+) and [^{14}C]DHA (○). Data are expressed as percentage of the cumulated counts of all fractions. [^{14}C]DHA standard was prepared by a short incubation of [^{14}C]ascorbate with ascorbate oxidase.

Formation of AFR

The oxidation of ascorbate by ascorbate oxidase results in the formation of ascorbate free radical, which can easily be detected by ESR spectroscopy. The ESR spectrum consists of a doublet with hyperfine splitting a^{H4} = 1.8 G (Fig. 4A). Control experiments showed that AFR peak intensities were constant for at least 15 min. The data in figure 4 were obtained with a scan time of 5 min and therefore represent steady state levels of AFR. No signal was observed when ascorbate was omitted from the incubation mixture (data not shown). Removal of ascorbate oxidase resulted in a small AFR signal, which amounted to 15% of the signal in figure 4A (data not shown). This signal was due to autoxidation and transition metal-mediated oxidation of ascorbate (36). Addition of erythrocytes decreased AFR signal intensity by 10%, whereas the addition of ascorbate-loaded erythrocytes decreased the AFR concentration considerably (compare Figs. 4, A, C and E).

In the presence of ascorbate-loaded erythrocytes, AFR could be generated both inside and outside the cell. To distinguish between intra- and extracellular AFR,

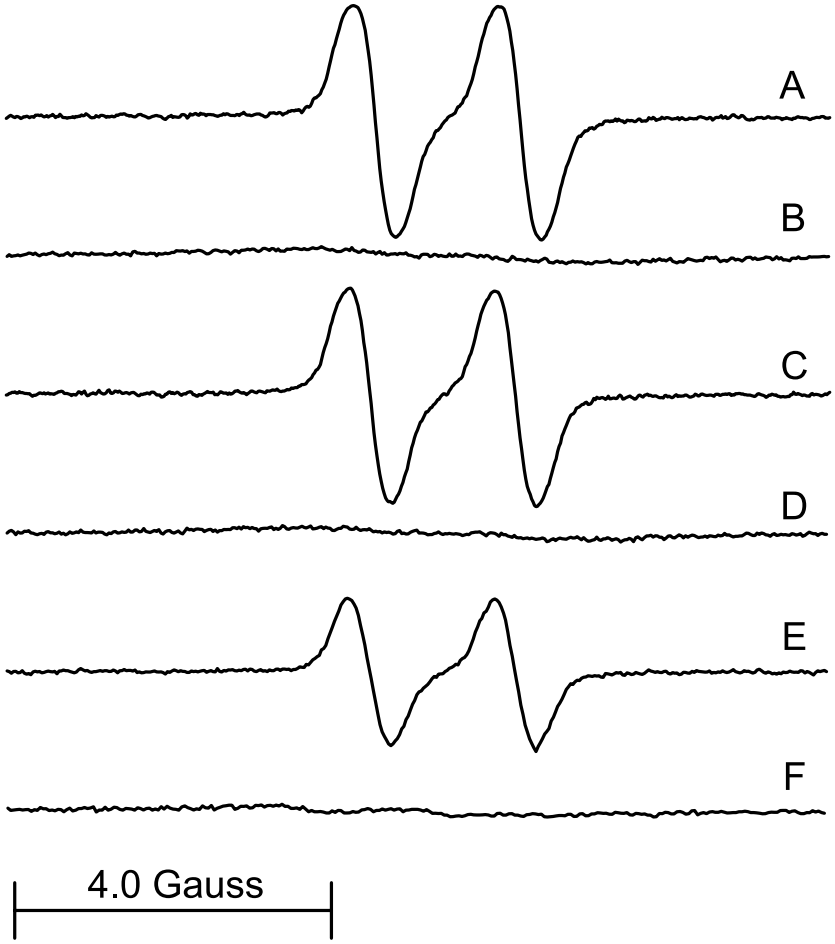

Figure 4. ESR spectra of ascorbate free radicals. A, 100 µM ascorbate and 4 mU/ml ascorbate oxidase; B, same as A, but with 5 mM $Ni(en)_3^{2+}$; C, same as A, but with 10% erythrocytes; D, same as B, but with 10% erythrocytes; E, same as A, but with 10 % ascorbate-loaded erythrocytes; F, same as B, but with 10 % ascorbate-loaded erythrocytes. Erythrocytes were loaded with ascorbate, using 500 µM DHA, as described under "Experimental Procedures".

the line broadening compound $Ni(en)_3^{2+}$ was used. This compound induces line broadening of the AFR signal, as is illustrated in figure 4B, without influencing its redox properties (37). Since $Ni(en)_3^{2+}$ cannot cross the plasma membrane, only the intensity of the extracellular AFR signal will be affected. Addition of 5 mM $Ni(en)_3^{2+}$ to an incubation of control or ascorbate-loaded erythrocytes in the presence of ascorbate and ascorbate oxidase, resulted in the line-broadening of the AFR signal (Figs. 5D and F). This shows that the AFR signal detected under these experimental conditions came from ascorbate radicals located outside the cell.

To determine the correlation between the intracellular ascorbate concentration and extracellular AFR, cells were preincubated with various DHA concentrations.

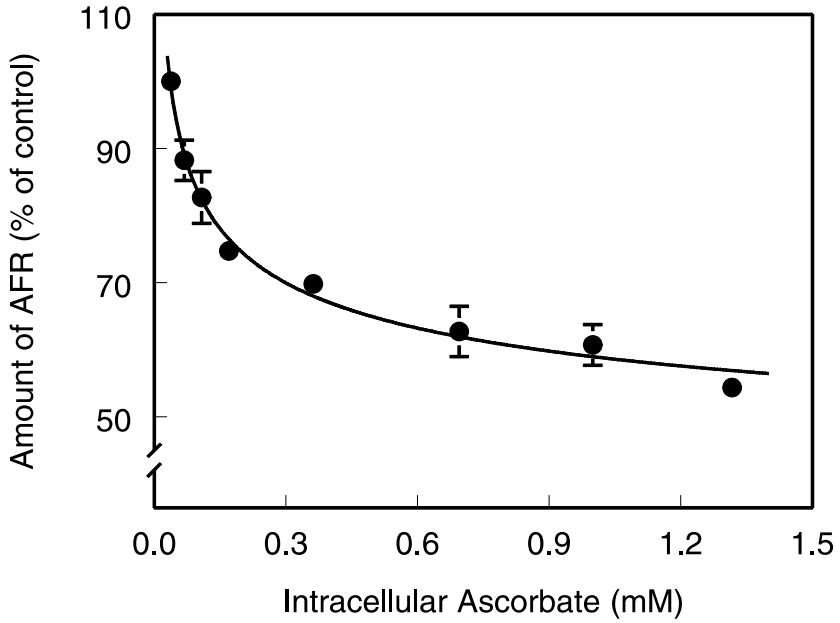

Figure 5. Correlation between the intracellular ascorbate concentration and the extracellular ascorbate free radical concentration. Experimental conditions were as described in figure 2. Immediately after addition of ascorbate and ascorbate oxidase, the samples were transferred to the flat cell, and ESR spectra were recorded and integrated. The levels are expressed, as an average ± S.E., relative to the amount of AFR found in the presence of unloaded erythrocytes.

Figure 5 shows the effect of increasing intracellular ascorbate concentrations on external AFR signal intensities. Ascorbate-loaded erythrocytes reduced the AFR signal in the extracellular medium in a concentration-dependent manner. The effect was similar to the effect observed in figure 2.

Formation of intracellular AFR

So far, our data seem to indicate that extracellular AFR is reduced by intracellular ascorbate. If the hypothesis is correct that intracellular ascorbate donates an electron to extracellular AFR, it can be expected that intracellular AFR is generated as a consequence of this reaction. The data in figure 4 suggest that under the present conditions, only extracellular AFR can be observed. The absence of an intracellular AFR signal is most likely due to the efficient regeneration of ascorbate in the erythrocyte. To overwhelm the reductive capacity of the cell, extracellular AFR levels were increased using high concentrations of ascorbate and ascorbate oxidase. Incubation of 1 mM ascorbate with 20 mU/ml ascorbate oxidase in the presence of 5 mM $Ni(en)_3^{2+}$ resulted in a broadened AFR signal (Fig. 6A). Subsequently, erythrocytes were added (20% suspension) and the spectrum in

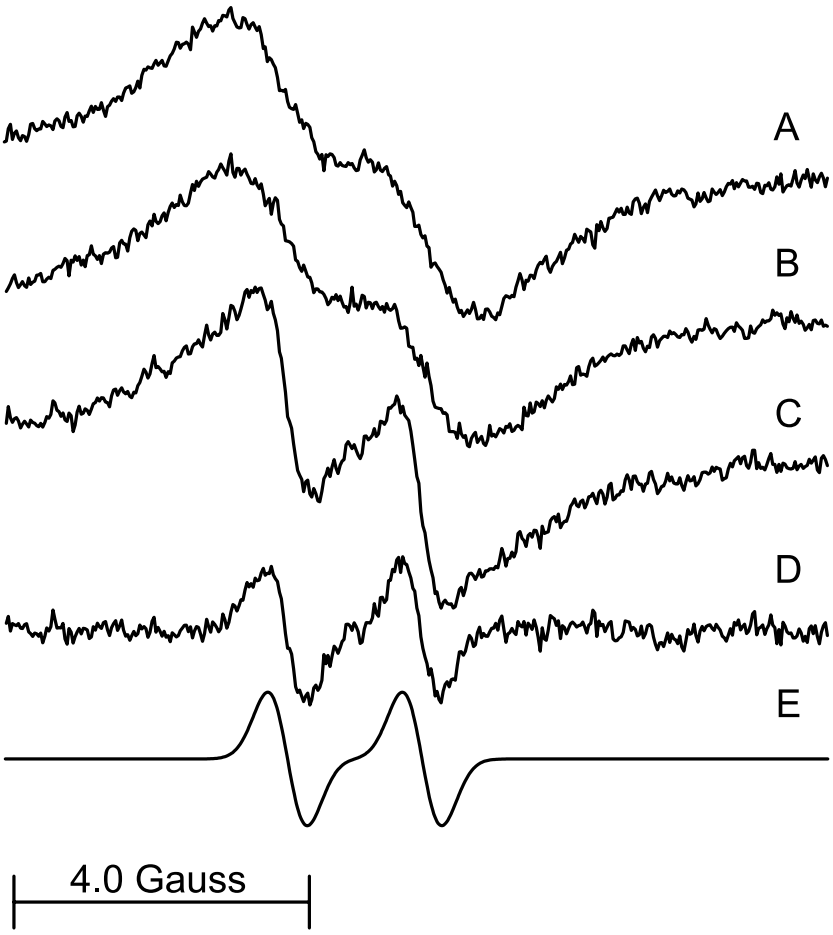

Figure 6. Detection of an intracellular AFR signal. A, ESR spectrum of 1 mM ascorbate, incubated with 20 mU/ml ascorbate oxidase in the presence of 5 mM $Ni(en)_3^{2+}$; B, same as A, but with 20% erythrocytes; C, same as A, but with 20% ascorbate-loaded erythrocytes; D, spectrum A was subtracted from spectrum C; E, simulation of D (hyperfine splitting constant a^{H4}=1.82 G; linewidth 0.27 G). Erythrocytes were loaded with ascorbate, using 500 µM DHA, as described in the Experimental section.

figure 6B was obtained, which is identical to the spectrum obtained without erythrocytes. When ascorbate-loaded erythrocytes were used, on the other hand, the ESR spectrum showed additional, slightly sharper peaks (Fig. 6C). This indicates that an additional AFR signal was formed that could not be broadened by $Ni(en)_3^{2+}$. Subtraction of curve A (Fig. 6) from C generated the spectrum given in figure 6D, which is typical for AFR in the absence of $Ni(en)_3^{2+}$ (simulated in Fig. 6E). This shows that at high levels of extracellular AFR, intracellular AFR can indeed be detected.

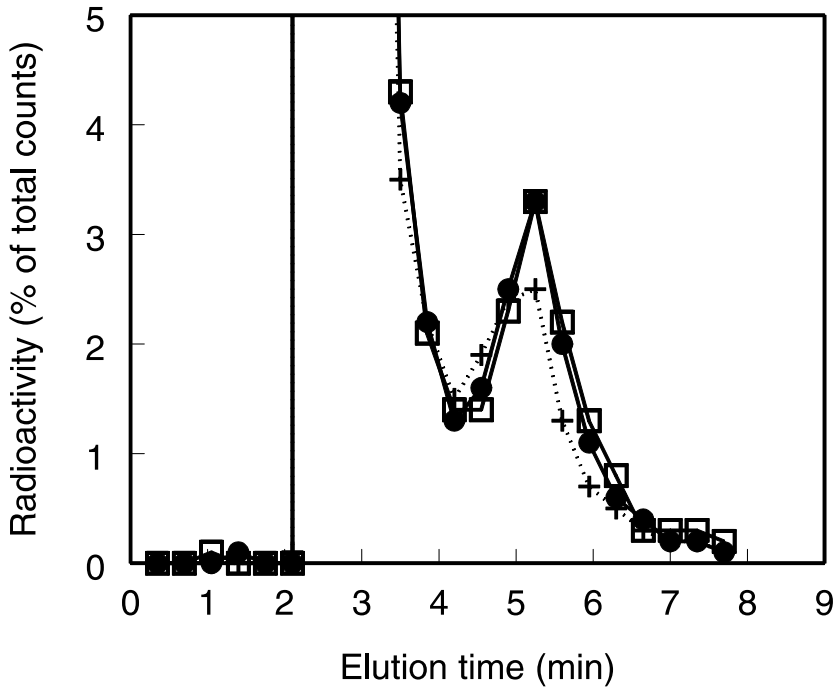

Figure 7. HPLC analysis of supernatants after incubation of [^{14}C]DHA in the presence or absence of erythrocytes. 1 mM [^{14}C]DHA, prepared with bromine as described in the Experimental section, was added to PBS (+) or to a 10% suspension of ascorbate-loaded (●) or control erythrocytes (□). After 10 min incubation at room temperature the samples were centrifuged, and the supernatant prepared for HPLC analysis as described in the Experimental section. Fractions were collected, and analyzed using liquid scintillation counting. In a control sample of [^{14}C]DHA at t=0, the peak eluting at 5.5 min was almost absent (not shown). Ascorbate elutes at 5.5 min, and DHA at 2.5 min. Data are expressed as percentage of the cumulated counts of all fractions.

Involvement of extracellular DHA

Although the ESR experiments indicated that extracellular AFR was reduced by the erythrocytes, we also investigated whether reduction of extracellular DHA played a role. [^{14}C]DHA was added either to PBS or to a 10% suspension of ascorbate-loaded or control erythrocytes. After 10 min, the supernatants were collected and injected on a HPLC system. A small peak at the retention time of ascorbate was found to develop during the incubation of DHA alone (Fig. 7). The presence of erythrocytes resulted in a slightly larger peak, but no differences were found between ascorbate-loaded and control erythrocytes.

Discussion

Addition of erythrocytes with increasing intracellular ascorbate concentrations decreased the oxidation rate of extracellular ascorbate by ascorbate oxidase (Fig. 1). At intracellular concentrations of 1 mM, ascorbate degradation was even completely prevented (Fig. 2). Removal of intracellular ascorbate by TEMPOL reversed the process (Fig. 1). In control experiments, inactivation of ascorbate oxidase and leakage of intracellular ascorbate could be eliminated as explanations for these findings. Thus, intracellular ascorbate directly influences the oxidation rate of extracellular ascorbate, even in the physiological range of 20-60 µM ascorbate (Fig. 2) (38). Addition of control erythrocytes, i.e. erythrocytes with only endogenous ascorbate, decreased the ascorbate oxidation with 30-40%. NADH also contributed to this effect, as the ascorbate oxidation rate was readily affected by changes in the concentration of NADH in the erythrocytes (Table 1). Unfortunately, the relative contribution of endogenous ascorbate and NADH could not be established experimentally, as depletion of endogenous ascorbate by TEMPOL also caused depletion of NADH (Table 1). Nevertheless, it can be concluded that the degradation of extracellular ascorbate is prevented by reactions that are driven by intracellular NADH and ascorbate. In erythrocytes loaded with ascorbate, the ascorbate-driven reaction prevails.

Ascorbate oxidase produces AFR from ascorbate in a direct reaction, followed by disproportionation of two AFR molecules to one molecule of ascorbate and one molecule of DHA (39). In order to restore ascorbate levels, either AFR or DHA has to be reduced to ascorbate. There have been reports on the reduction of extracellular DHA by K562 cells (40) and by erythrocytes (21), but our data did not indicate an important role for this process. A small amount of ascorbate or an ascorbate-like compound was formed on incubation of DHA in PBS (Fig. 7). Jung and Wells described a degradation product of DHA that can reduce DHA to ascorbate (41). In addition, degradation of DHA results in erythroascorbic acid, an analogue of ascorbate that could elute very similar in our HPLC system. These processes could contribute to the formation of the ascorbate-like peak at 5.5 min. The peak increased slightly in the presence of erythrocytes, irrespective of the presence of intracellular ascorbate. This small increase may be caused by a NADH-dependent redox system. Thus, although our data indicate that erythrocytes reduce some extracellular DHA, it is evident that this does not depend on the intracellular ascorbate concentration.

Reduction of ascorbate radical levels could be established using ESR spectroscopy (Figs. 4 and 5). A correlation was found between increasing intracellular ascorbate concentrations and the decrease in extracellular ascorbate oxidation, reflected

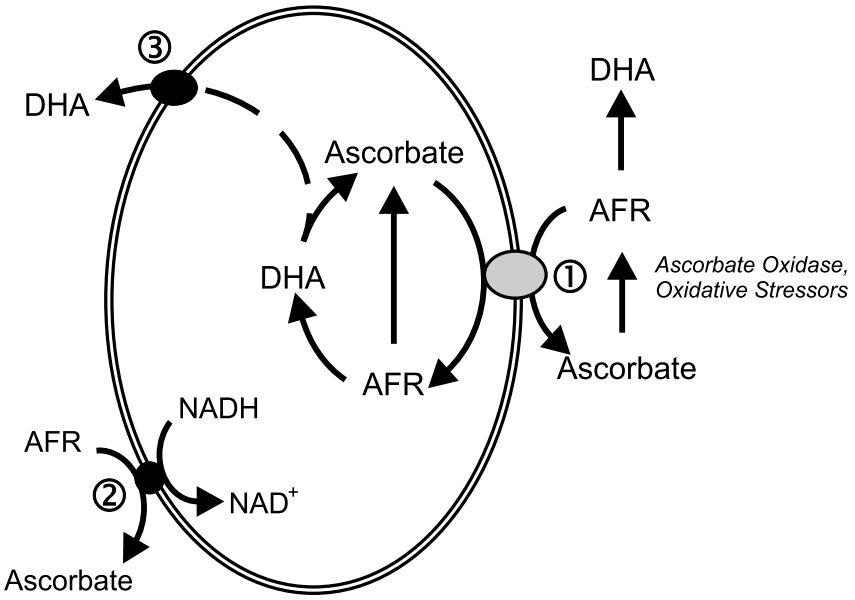

Figure 8. Model for the mechanism of the reduction of extracellular AFR by intracellular ascorbate. Extracellular AFR is reduced by intracellular ascorbate via an ascorbate:AFR reductase [1], as discussed in this chapter. AFR can also be reduced by an NADH-dependent AFR reductase [2]. The efflux of DHA from erythrocytes is shown as a dashed line through the GLUT-1 transporter [3].

either by the absorbance of ascorbate in the extracellular medium or by steady state levels of AFR (Figs. 2 and 5). This suggests that there is a close relationship between the intracellular ascorbate concentration and the reduction of extracellular oxidation products. A plausible explanation is that intracellular ascorbate supplies an electron that reduces extracellular AFR to extracellular ascorbate. In this process, intracellular ascorbate will be oxidized to an ascorbate radical. Either this radical can be reduced to ascorbate via intracellular NADH-AFR reductases or two radicals can disproportionate to ascorbate and DHA. At first, we were unable to detect intracellular AFR. This can be explained by the fact that with low levels of extracellular AFR the cell is able to regenerate intracellular ascorbate efficiently, and intracellular levels of AFR will be too low to be detected. Higher levels of AFR will cause a more severe oxidative stress over the plasma membrane and intracellular regeneration of ascorbate might be overwhelmed. This was indeed the case. Using $Ni(en)_3^{2+}$ to line broaden extracellular AFR signals, a small intracellular AFR signal could be detected (Fig. 6). This strongly suggests that intracellular ascorbate donates an electron to extracellular AFR, forming extracellular ascorbate and intracellular AFR.

The efflux of ^{14}C-labeled material from the erythrocytes provided additional evidence (Fig. 3 and Table 2). HPLC analysis of the extracellular medium revealed that ^{14}C-labeled material emerged as [^{14}C]DHA, rather than [^{14}C]ascorbate (Fig. 3). The efflux of ^{14}C-labeled material was increased in the presence of ascorbate and ascorbate oxidase. This confirmed that external AFR induced an oxidative stress over the membrane, which resulted in the oxidation of intracellular ascorbate. At high levels of AFR, the production of DHA exceeds the capacity of the cell for regeneration to ascorbate, resulting in efflux of DHA via the GLUT-1 transporter (Table 2).

Although there is good evidence for ascorbate-dependent electron transport across the membrane, it is still not clear what the functional components of this system are. Possible mechanisms are transport chains of a physico-chemical nature, e.g. an α-tocopherol electron shuttle, and protein-mediated systems such as an integral plasma membrane protein, complexes of multiple proteins, or proteins using a cofactor to traverse the membrane. However, the standard reduction potentials (E'_0) of α-tocopherol and ascorbate with their respective radicals differ more than 200 mV, which makes the transfer of an electron from α-tocopherol to AFR unlikely (1). Another putative co-factor is coenzyme Q, but its capability to move freely across the lipid bilayer has been questioned (27, 42). Moreover, capsaicin and dicumarol, inhibitors of coenzyme Q mediated electron transport, did not inhibit the decrease in the level of AFR in the presence of ascorbate-loaded erythrocytes (data not shown). The most likely form appears to be a single protein or a protein complex, accepting electrons from intracellular ascorbate and transporting them to extracellular AFR. Interestingly, a cytochrome with a similar function has been described in chromaffin cells from the adrenal medulla. In chromaffin vesicles inside these cells norepinephrine is synthesized at the expense of large amounts of ascorbate. A cytochrome b_{561} is present in the vesicle membrane, transporting electrons from cytoplasmic ascorbate to intravesicular AFR (43, 44). A similar or identical cytochrome could explain the ascorbate:AFR oxidoreductase activity in the erythrocyte. Indeed, evidence was found for the presence of a *b*-cytochrome in the erythrocyte membrane with spectral characteristics similar to those of cytochrome b_{561}. However, it has been reported that cytochrome b_{561} is not present in the erythrocyte (45). This was confirmed in recent work by our group. It is conceivable that a cytochrome similar to cytochrome b_{561} is responsible for the reduction of AFR by the erythrocyte, but so far, no proteins with structural homology to cytochrome b_{561} have been described.

The various processes in the erythrocyte are summarized in figure 8. Extracellular AFR is reduced by intracellular ascorbate via an ascorbate:AFR reductase [1]. Concurrently, intracellular ascorbate is oxidized to AFR, which can be reduced to ascorbate via intracellular NADH-AFR reductases or disproportionate to ascorbate and DHA. Efflux of DHA proceeds through the GLUT-1 transporter [3], which facilitates bi-directional transport of DHA over the plasma membrane. In addition, extracellular AFR can be reduced by a NADH-dependent AFR reductase [2]. Presently, it is not known whether the ascorbate-dependent AFR reductase discussed in this chapter is related in any way to other AFR reductases known from the literature. Generation of intracellular AFR, as well as efflux of DHA, was found by May et al. upon incubation of erythrocytes with ferricyanide (11, 29). However, recently May and Qu observed that pCMBS partly inhibited ascorbate-dependent ferricyanide reduction in erythrocytes, whereas it did not influence ferric iron reduction (46). We were unable to find any effect of *p*CMBS on the AFR reductive capacity (data not shown). This suggests that AFR and ferricyanide are not reduced by the same reductase, but could imply that the erythrocyte AFR reductase also catalyzes ferric iron reduction. Further studies are, however, required to identify the transmembrane reductase or reductases that are responsible for the ascorbate-dependent reduction of extracellular oxidants.

References

1. Buettner GR. The pecking order of free radicals and antioxidants: lipid peroxidation, α-tocopherol, and ascorbate. *Arch. Biochem. Biophys.* 1993; 300:535-543.

2. Rose RC, Bode AM. Biology of free radical scavengers: an evaluation of ascorbate. *FASEB J* 1993; 7:1135-1142.

3. Padh H. Cellular functions of ascorbic acid. *Biochem Cell Biol* 1990; 68:1166-1173.

4. Gershoff SN. Vitamin C (ascorbic acid): new roles, new requirements? *Nutr Rev* 1993; 51:313-326.

5. Block G. Epidemiologic evidence regarding vitamin C and cancer. *Am J Clin Nutr* 1991; 54:1310s-1314s.

6. Bianchi J, Rose RC. Dehydroascorbic acid and cell membranes: possible disruptive effects. *Toxicology* 1986; 40:75-82.

7. Pence LA, Mennear JH. Inhibition effect of dehydroascorbic acid on insulin secretion from mouse pancreatic islets. *Toxicol Appl Pharmacol* 1979; 50:57-65.

8. Rose RC, Choi JL *et al*. Short term effects of oxidized ascorbic acid on bovine corneal endothelium and human placenta. *Life Sci* 1992; 50:1543-1549.

9. Van Duijn MM, Van der Zee J *et al*. Ascorbate stimulates ferricyanide reduction in HL-60 cells through a mechanism distinct from the NADH-dependent plasma membrane reductase. *J Biol Chem* 1998; 273:13415-13420.

10. Bode AM, Cunningham L *et al*. Spontaneous decay of oxidized ascorbic acid (dehydro-L-ascorbic acid) evaluated by high-pressure liquid chromatography. *Clin Chem* 1990; 36:1807-1809.

11. May JM, Qu ZC *et al*. Ascorbate recycling in human erythrocytes: role of GSH in reducing dehydroascorbate. *Free Radic Biol Med* 1996; 20:543-551.

12. May JM, Mendiratta S *et al*. Reduction of dehydroascorbate to ascorbate by the selenoenzyme thioredoxin reductase. *J Biol Chem* 1997; 272:22607-22610.

13. May JM, Cobb CE *et al*. Reduction of the ascorbyl free radical to ascorbate by thioredoxin reductase. *J Biol Chem* 1998; 273:23039-23045.

14. Mendiratta S, Qu ZC *et al*. Enzyme-dependent ascorbate recycling in human erythrocytes: Role of thioredoxin reductase. *Free Radic Biol Med* 1998; 25:221-228.

15. Park JB, Levine M. Purification, cloning and expression of dehydroascorbic acid-reducing activity from human neutrophils: identification as glutaredoxin. *Biochem J* 1996; 315:931-938.

16. Gilbert HF. Protein disulfide isomerase. *Methods Enzymol* 1998; 290:26-50.

17. Diliberto EJ, Jr., Dean G *et al*. Tissue, subcellular, and submitochondrial distributions of semidehydroascorbate reductase: possible role of semidehydroascorbate reductase in cofactor regeneration. *J Neurochem* 1982; 39:563-568.

18. Vera JC, Rivas CI *et al*. Resolution of the facilitated transport of dehydroascorbic acid from its intracellular accumulation as ascorbic acid. *J Biol Chem* 1995; 270:23706-23712.

19. Rumsey SC, Kwon O *et al*. Glucose transporter isoforms GLUT1 and GLUT3 transport dehydroascorbic acid. *J Biol Chem* 1997; 272:18982-18989.

20. Goldenberg H, Schweinzer E. Transport of vitamin C in animal and human cells. *J Bioenerg Biomembr* 1994; 26:359-367.

21. Himmelreich U, Drew KN *et al*. C-13 NMR studies of vitamin C transport and its redox

cycling in human erythrocytes. *Biochemistry* 1998; 37:7578-7588.

22. Villalba JM, Canalejo A *et al*. NADH-ascorbate free radical and -ferricyanide reductase activities represent different levels of plasma membrane electron transport. *J Bioenerg Biomembr* 1993; 25:411-417.

23. May JM, Qu ZC *et al*. Extracellular reduction of the ascorbate free radical by human erythrocytes. *Biochem Biophys Res Commun* 2000; 267:118-123.

24. Goldenberg H, Grebing C *et al*. NADH-monodehydroascorbate reductase in human erythrocyte membranes. *Biochem Int* 1983; 6:1-9.

25. May JM, Qu ZC *et al*. Ascorbate is the major electron donor for a transmembrane oxidoreductase of human erythrocytes. *Biochim. Biophys. Acta* 1995; 1238:127-136.

26. Orringer EP, Roer MES. An ascorbate-mediated transmembrane-reducing system of the human erythrocyte. *J Clin Invest* 1979; 63:53-58.

27. May JM. Is ascorbic acid an antioxidant for the plasma membrane? *FASEB J* 1999; 13:995-1006.

28. State HM. Tris(ethylenediamine)Nickel(II)chloride 2-hydrate Trispropylenediamine)Nickel(II)chloride 2-hydrate. *Inorganic Syntheses* 1960; 6:200-201.

29. May JM, Qu ZC *et al*. Ascorbic acid recycling enhances the antioxidant reserve of human erythrocytes. *Biochemistry* 1995; 34:12721-12728.

30. May JM, Qu ZC *et al*. Protection and recycling of α-tocopherol in human erythrocytes by intracellular ascorbic acid. *Arch Biochem Biophys* 1998; 349:281-289.

31. Mehlhorn RJ. Ascorbate- and dehydroascorbic acid-mediated reduction of free radicals in the human erythrocyte. *J Biol Chem* 1991; 266:2724-2731.

32. Wagner TC, Scott MD. Single extraction method for the spectrophotometric quantification of oxidized and reduced pyridine nucleotides in erythrocytes. *Anal Biochem* 1994; 222:417-426.

33. Washko PW, Wang Y *et al*. Ascorbic acid recycling in human neutrophils. *J Biol Chem* 1993; 268:15531-15535.

34. Alvarez J, Camaleno JM *et al*. Modulation of Ca^{2+}-dependent K^+ transport by modifications of the NAD^+/NADH ratio in intact human red cells. *Biochim Biophys Acta* 1986; 856:408-411.

35. Momsen G. Effect of increasing the intracellular ratio of NADH to NAD^+ on human erythrocyte metabolism: new estimation of the turnover through the phosphoglycerate shunt. *Arch Biochem Biophys* 1981; 210:160-166.

36. Van Duijn MM, Van der Zee J *et al*. Electron spin resonance study on the formation of ascorbate free radical from ascorbate: the effect of dehydroascorbic acid and ferricyanide. *Protoplasma* 1998; 205:122-128.

37. Wakefield LM, Cass AE *et al*. Electron transfer across the chromaffin granule membrane. Use of EPR to demonstrate reduction of intravesicular ascorbate radical by the extravesicular mitochondrial NADH:ascorbate radical oxidoreductase. *J Biol Chem* 1986; 261:9746-9752.

38. Evans RM, Currie L *et al*. The distribution of ascorbic acid between various cellular components of blood, in normal individuals, and its relation to the plasma concentration. *Br J Nutr* 1982; 47:473-482.

39. Yamazaki I, Piette LH. Mechanism of free radical formation and disappearance during the

ascorbic acid oxidase and peroxidase reactions. *Biochim Biophys Acta* 1961; 50:62-69.

40. Schweinzer E, Mao Y *et al*. Reduction of extracellular dehydroascorbic acid by K562 cells. *Cell Biochem Funct* 1996; 14:27-31.

41. Jung CH, Wells WW. Spontaneous conversion of L-dehydroascorbic acid to L-ascorbic acid and L-erythroascorbic acid. *Arch Biochem Biophys* 1998; 355:9-14.

42. Ulrich EL, Girvin ME *et al*. Location and mobility of ubiquinones of different chain lengths in artificial membrane vesicles. *Biochemistry* 1985; 24:2501-2508.

43. Srivastava M, Duong LT *et al*. Cytochrome b_{561} catalyzes transmembrane electron transfer. *J Biol Chem* 1984; 259:8072-8075.

44. Wakefield LM, Cass AE *et al*. Functional coupling between enzymes of the chromaffin granule membrane. *J Biol Chem* 1986; 261:9739-9745.

45. Pruss RM, Shepard EA. Cytochrome b_{561} can be detected in many neuroendocrine tissues using a specific monoclonal antibody. *Neuroscience* 1987; 22:149-157.

46. May JM, Qu ZC. Ascorbate-dependent electron transfer across the human erythrocyte membrane. *Biochim Biophys Acta* 1999; 1421:19-31.