
CHAPTER 3

Ascorbate stimulates ferricyanide reduction in HL60 cells through a mechanism distinct from the NADH-dependent plasma membrane reductase

This chapter was adapted from MM Van Duijn, J Van der Zee, J VanSteveninck, and PJA Van den Broek, Ascorbate stimulates ferricyanide reduction in HL-60 cells through a mechanism distinct from the NADH-dependent plasma membrane reductase. J Biol Chem, 273(22): 13415-13420, 1998

Summary

The non-permeating oxidant ferricyanide is reduced by the plasma membrane redox system of HL60 cells. The rate of reduction is strongly enhanced by ascorbate or dehydroascorbate. The aim of this study was to determine the mechanism by which ascorbate and dehydroascorbate accelerate ferricyanide reduction in HL60 cells. Addition of ascorbate or dehydroascorbate to cells in the presence of ferricyanide led to the intracellular accumulation of ascorbate. Control experiments showed that extracellular ascorbate was rapidly converted to dehydroascorbate in the presence of ferricyanide. These data suggest that intracellular ascorbate originates from extracellular dehydroascorbate. Accumulation of ascorbate was prevented by inhibitors of dehydroascorbate transport into the cell. These compounds also strongly inhibited ascorbate-stimulated ferricyanide reduction in HL60 cells. Thus it is concluded that the stimulation of ferricyanide reduction is dependent on intracellular accumulation of ascorbate. Changing the α -tocopherol content of the cells had no effect on the ascorbate-stimulated ferricyanide reduction, showing that a non-enzymatic redox system utilizing α -tocopherol was not involved. *para*-(Chloromercuri)benzenesulfonic acid strongly affected ferricyanide reduction in the absence of ascorbate, whereas the stimulated reaction was much less responsive to this compound. Thus, it appeared that at least two different membrane redox systems are operative in HL60 cells, both capable of reducing ferricyanide, but through different mechanisms. The first system is the ferricyanide-reductase, which uses NADH as its source for electrons, while the novel system proposed in this chapter relies on ascorbate.

Introduction

Many eukaryotic cells contain a redox system in their plasma membrane, capable of reducing extracellular substrates using electrons from intracellular NADH (1). The system efficiently reduces the impermeable substrate ferricyanide. This is not the natural substrate for the redox system, but as yet there has been no conclusive evidence for the substrates of this system or for its primary function. It has been suggested that the system is involved in the maintenance of the redox state of SH-residues in membrane proteins (1), the neutralization of oxidative stressors outside the cell (2) or in the uptake of iron through a non-transferrin pathway (3, 4).

This redox system deserves special attention because of its possible involvement in regulation of growth and differentiation. Activation of the redox system results

in stimulation of growth in serum-limited HeLa cells (5) and HL60 cells (6). Induction of differentiation in HL60 cells has been associated with transient changes in reductase activity (7). In other cells, activation of the redox system has been shown to modulate protein kinase C activity (8). These findings raise an interest in the role of the plasma membrane reductase in these cells, and the mechanisms through which it operates.

The reduction of ferricyanide by the plasma membrane reductase can be greatly stimulated by the addition of ascorbate and the oxidized form of ascorbate, dehydroascorbate (DHA). This has been found both in K562 cells (9), a leukemic cell line, and in human erythrocytes (10). Although several mechanisms have been proposed, the exact mechanism of this enhancement by ascorbate and DHA remains to be elucidated. For K562 cells, it was suggested that the stimulation of ferricyanide reduction by ascorbate was due to a plasma membrane-localized ascorbate free radical (AFR) reductase (9). This enzyme is supposed to catalyze the reduction of external ascorbate free radical using intracellular NADH. It was proposed that ferricyanide reacts with ascorbate to ferrocyanide and the ascorbate free radical. The latter would then be regenerated by the AFR-reductase to ascorbate, which can subsequently again react with ferricyanide.

Another mechanism is based on studies on erythrocytes and involves the accumulation of ascorbate in cells, where it may serve as an intracellular electron donor for a plasma membrane reductase (10). In many cells, accumulation of ascorbate is achieved through a facilitative glucose transporter, GLUT-1 (11, 12). This transporter efficiently transports DHA into the cell, but not ascorbate itself. Inside the cell, DHA is reduced to ascorbate. This metabolic trapping mechanism enables the cell to accumulate ascorbate at concentrations far exceeding that of its environment. However, recently, it was found that in erythrocytes, the enhancement of ferricyanide reduction by DHA was not affected by an inhibitor of the GLUT-1 transporter (13). This led to the conclusion that ascorbate was closely involved in the redox reaction, but that it acted both intra- and extracellularly. It was concluded that DHA could be regenerated to ascorbate independent of its cellular location. Thus, controversy exists on the mechanism of enhancement of ferricyanide reduction by ascorbate and DHA.

We have studied the effect of DHA and ascorbate on ferricyanide reduction by HL60 cells. It has been reported that HL60 cells contain a ferricyanide reductase and an AFR-reductase (14, 15). In this study, the possible role of both enzymes in the enhanced ferricyanide reduction was investigated. It is concluded that ascorbate-stimulated ferricyanide reduction does not involve an AFR-reductase.

Rather, it involves a mechanism where ascorbate is internalized and where intracellular ascorbate is involved in the reduction of extracellular ferricyanide by a novel redox system, different from the NADH-driven ferricyanide reductase.

Experimental Procedures

L-Ascorbic acid and L-dehydroascorbic acid were purchased from Aldrich, and potassium ferricyanide and *para*-(chloromercuri)benzenesulfonic acid (*p*CMBS) from Fluka. Cytochalasin B and α -tocopherol were from Sigma, L-buthionine-(RS)-sulfoximine (BSO) from Acros Chimica and phloretin from ICN.

HL60 myeloid leukemic cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Life Technologies, Inc.), 100 U/ml penicillin G, 100 μ g/ml streptomycin, 2.5 μ g/ml amphotericin B and 3 mM L-glutamine. Culture flasks were kept in a humidified atmosphere with 5% CO₂ at 37 °C. Cells were harvested when the culture had reached a density of $\sim 9 \cdot 10^5$ cells/ml, and were washed twice in 20 mM Tris/150 mM NaCl at pH 7.4 (Tris/NaCl). Subsequently, the cells were suspended in this buffer for further use.

Ferricyanide reduction was determined as follows: $4 \cdot 10^6$ cells/ml in Tris/NaCl were incubated at 37 °C in a closed shaking waterbath. The reaction was started by the addition of 1 mM ferricyanide to suspensions containing various concentrations of ascorbate or DHA, and was followed for at least 60 min. Aliquots were centrifuged, and supernatants were assayed for ferrocyanide using the bathophenanthroline disulphonic acid assay (16). The rate of ferrocyanide generation was used as a measure for the activity of the reductase.

Accumulation of ascorbate in cells was determined by incubating $4 \cdot 10^6$ cells/ml in Tris/NaCl in the presence of ascorbate or DHA, with or without 1 mM ferricyanide. The suspension was incubated at 37 °C in a closed shaking waterbath, and 1 ml aliquots were taken at set time points. After centrifugation, cells were washed twice in ice-cold Tris/NaCl, containing 100 μ M of the GLUT-1 inhibitor phloretin (11, 13). The cell pellet was extracted with 600 μ l methanol, which was diluted to 1 ml with water and 1 mM EDTA (end-concentration). After centrifugation, the supernatants were analyzed for ascorbate on an HPLC system with a Partisil SAX column (10 μ m, 250 x 4.6 mm), eluting with a gradient starting at 7 mM potassium phosphate, pH 4.0, and changing to 0.25 M potassium phosphate, 0.5 M KCl, pH 5.0. Ascorbate was detected at 265 nm using a Jasco 870-UV detector. Extraction and HPLC analysis were validated using [¹⁴C]-ascorbate (Amersham). The extraction

yielded over 95 % of the cell-associated radioactivity, and HPLC analysis revealed that 90% of intracellular radioactivity corresponded with ascorbate.

The stability of ascorbate and DHA was tested by incubating 25 μM of either compound with $4 \cdot 10^6$ cells/ml in Tris/NaCl, with or without 1 mM ferricyanide at 37 °C in a closed shaking waterbath. At set times 100 μl aliquots were mixed with 50 μl 0.1 M dithiothreitol (DTT), 600 μl methanol and 250 μl water. The extracts were spun down, and the ascorbate concentration in the supernatant was determined by HPLC.

Glutathione (GSH/GSSG) depletion was achieved by incubating cells with BSO (17). Cells were incubated at a density of $3.5 \cdot 10^5$ /ml in growth medium supplemented with 0 or 500 μM BSO. After 2 days at 37 °C, cells were centrifuged and washed twice with Tris/NaCl. Glutathione levels in cells, i.e. the total amount of GSH/GSSG, were measured using an enzyme cycling assay (18).

α -Tocopherol depletion was achieved by growing cells for at least six generations in medium, where fetal bovine serum was replaced by 5 $\mu\text{g/ml}$ transferrin, 5 $\mu\text{g/ml}$ insulin and 0.5% bovine serum albumin (19). α -Tocopherol supplementation of cells was achieved by growing cells for 2 days at 37 °C in culture medium, supplemented with 0, 30 or 100 μM α -tocopherol, at an initial cell density of $3.5 \cdot 10^5$ cells/ml.

α -Tocopherol was extracted and analyzed essentially as described by Thurnham *et al.* (20). Briefly, $5 \cdot 10^6$ cells were spun down and lysed with 10 mM SDS and ethanol containing α -tocopherol-acetate as an internal standard. The lysate was extracted using heptane, which was subsequently evaporated under N_2 . The samples were dissolved in the mobile phase of the HPLC system (methanol/acetonitrile/chloroform 47/47/6 (v/v)), injected on a Spherisorb ODS-2 column (250 x 4.6 mm), and eluted isocratically. Both α -tocopherol and α -tocopherol-acetate were detected at 292 nm.

ESR spectra were recorded on a JEOL-RE2X spectrometer operating at 9.36 GHz with a 100 kHz modulation frequency, equipped with a TM_{110} cavity. Samples were transferred to a quartz flat cell using a sampling device, which allowed sampling within seconds after mixing. ESR spectrometer settings were as follows: microwave power, 40 mW; modulation amplitude, 1 G; time constant, 0.1 s; scan rate, 6 G/min.

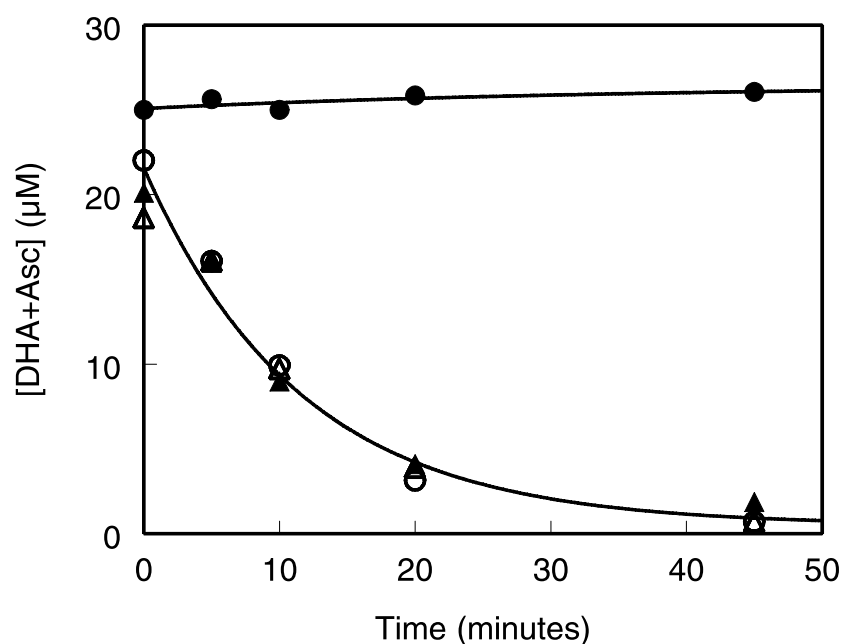


Figure 1: Stability of ascorbate and DHA. 25 μ M DHA (▲), 25 μ M DHA + 1 mM ferricyanide (△), 25 μ M ascorbate (●) or 25 μ M ascorbate + 1 mM ferricyanide (○) were incubated in Tris/NaCl in the presence of $4 \cdot 10^6$ cells/ml. Samples were taken and processed as described in the Experimental Procedures. Results represent total ascorbate (Asc) plus DHA concentration.

Results

Ascorbate and DHA metabolism

Several papers mention the rapid irreversible degradation of DHA in solution (11, 21), yielding 2,3-diketo-L-gulonic acid (22). Therefore, the stability of ascorbate and DHA in cell suspensions was first determined. DHA can be measured by addition of dithiothreitol, which converts DHA to ascorbate, after which the ascorbate concentration can be determined by its absorbance at 256 nm. Samples were mixed with DTT and methanol in order to determine the total, i.e. intra- and extracellular, amount of ascorbate and DHA. As expected it was found that DHA was rapidly degraded in a suspension at pH 7.4 (Fig. 1), with a half-life of 8-9 min. Ascorbate remained stable for at least 90 min. Ferricyanide had no effect on the rate of DHA degradation (Fig. 1). Addition of ferricyanide to ascorbate, on the other hand, resulted in a rapid decrease in ascorbate, with similar kinetics as found for DHA.

The data in figure 1 show that a residual amount of ascorbate or DHA can be observed after 45 min and remains relatively stable. HPLC analysis revealed that

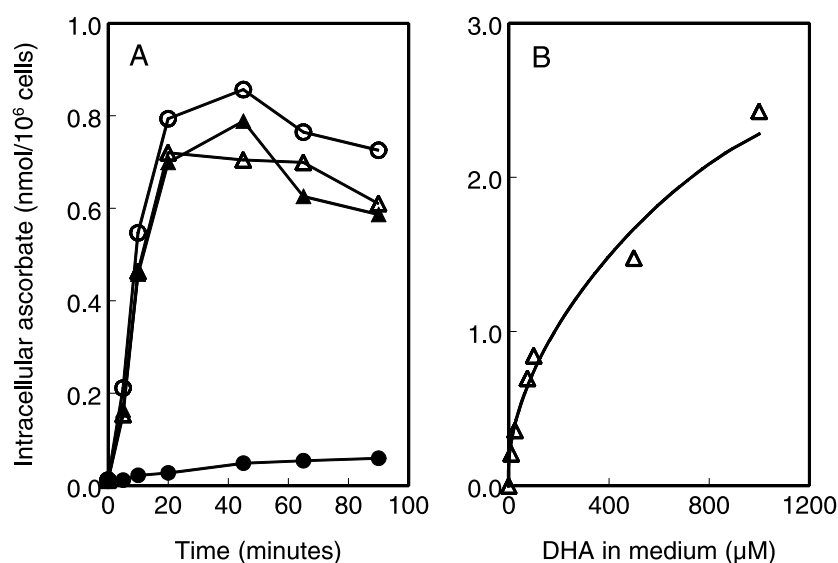


Figure 2: Accumulation of ascorbate in HL60 cells. (A) uptake after incubation with 25 μ M DHA (▲), 25 μ M DHA + 1 mM ferricyanide (△), 25 μ M ascorbate (●) and 25 μ M ascorbate + 1 mM ferricyanide (○). (B) ascorbate accumulated after 20 min incubation with various concentrations of DHA.

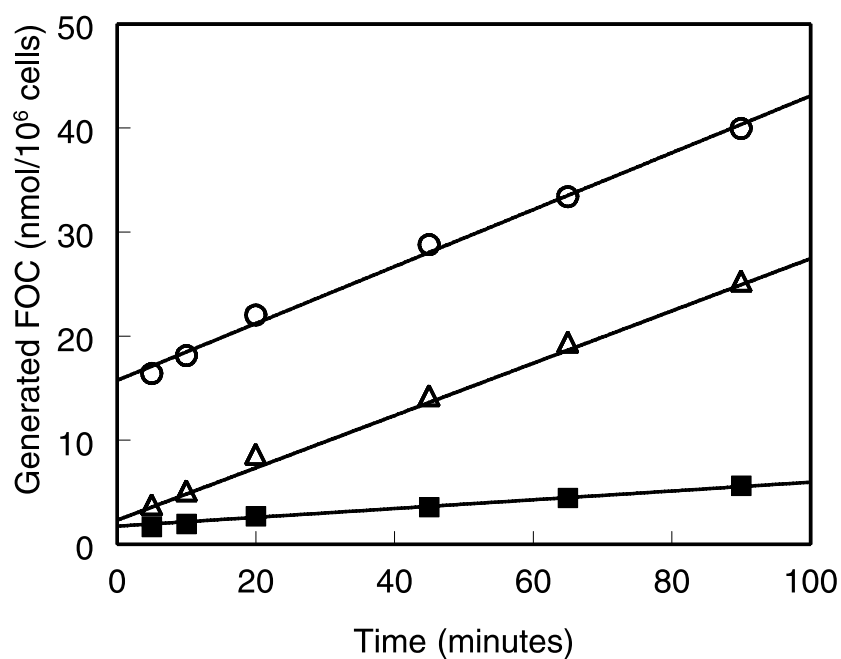


Figure 3: Ferricyanide reduction in HL60 cells. Cells were incubated with 1 mM ferricyanide (■), 25 μ M DHA and 1 mM ferricyanide (△) or 25 μ M ascorbate and 1 mM ferricyanide (○). Ferricyanide reduction was determined as described in the Experimental Procedures. FOC is ferrocyanide.

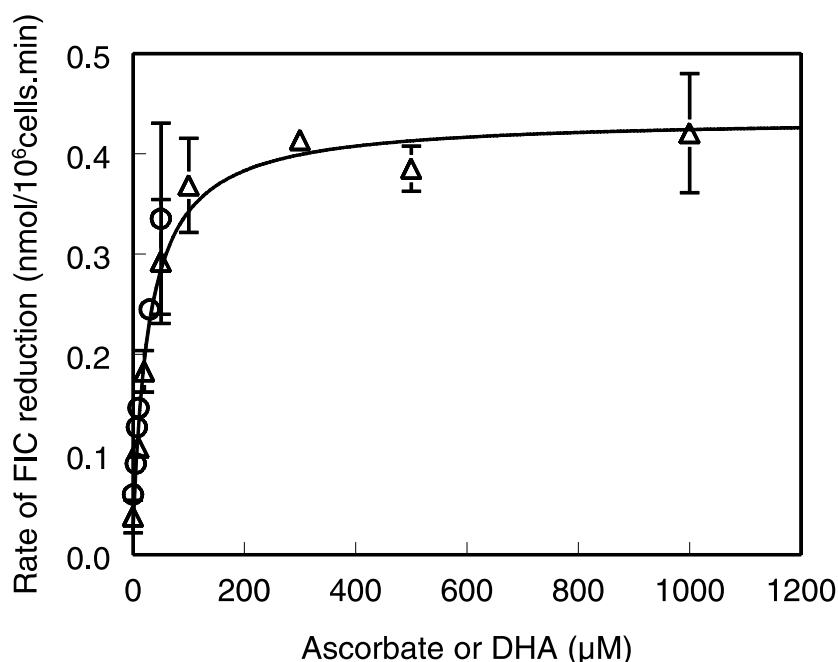


Figure 4: Dependence of the ferricyanide reduction rate on the concentration of ascorbate or DHA. Ferricyanide (FIC) reduction was measured in the presence of various concentrations of ascorbate (○) or DHA (Δ). Error bars represent the standard deviation.

this residue was ascorbate (data not shown) and corresponded with ascorbate accumulated in the cells (Fig. 2A). Cells incubated with ascorbate alone accumulated only a small amount of ascorbate. However, cells incubated with DHA, DHA and ferricyanide, or ascorbate and ferricyanide rapidly accumulated ascorbate, reaching maximum levels after 20 min of incubation (Fig. 2A). The maximum of approximately 0.8 nmol/10⁶ cells corresponded with an intracellular concentration of 1.5 mM (assuming a cell diameter of 10 μm). After 45 min, the levels of ascorbate showed a slight decrease, though most of it remained in the cells. The accumulation of ascorbate proved to be dependent on the concentration of DHA in the incubation mixture (Fig. 2B).

Enhancement of ferricyanide reduction

Subsequently, the effect of ascorbate and DHA on ferricyanide reduction by HL60 cells was investigated. Without ascorbate and DHA, the reduction of ferricyanide to ferrocyanide was linear for at least 90 min (Fig. 3). In the presence of ascorbate and DHA, the rate of reduction was higher and the same linear kinetics were found for both compounds. It should be noted that, with ascorbate, a sudden increase in ferrocyanide levels could be observed within seconds after mixing of ferricyanide and ascorbate (Fig. 3). This increase, which also occurred in the absence

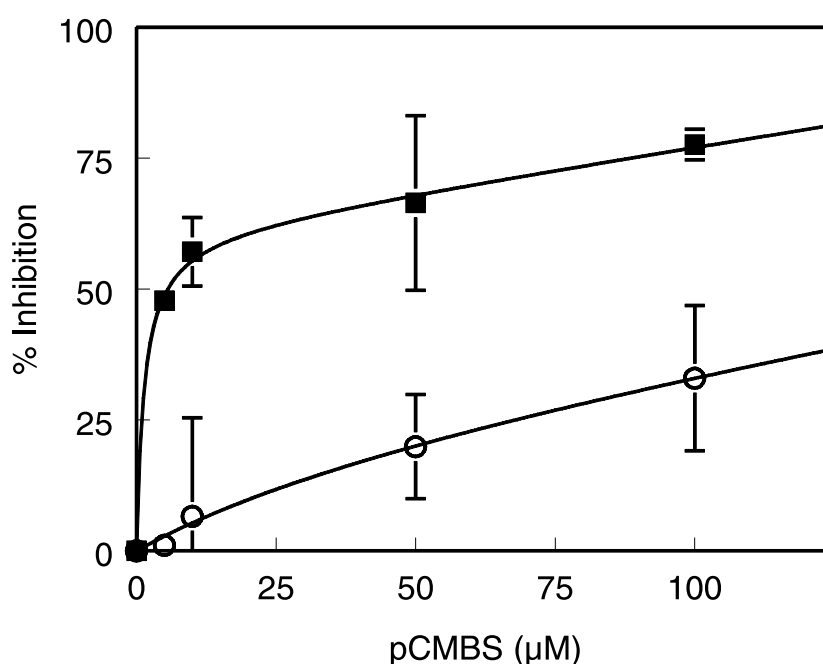


Figure 5: The effect of *p*CMBS on ferricyanide reduction. *p*CMBS was added at the indicated concentration 15 min before adding 1 mM ferricyanide (■) or 1 mM ferricyanide and 25 μM ascorbate (○). The effect of *p*CMBS is expressed as % inhibition of the control without *p*CMBS. Control ferricyanide reduction rates without and with ascorbate were 29.7 and 199.3 pmol/10⁶ cells·min respectively. Error bars represent the standard deviation.

of cells, had a stoichiometry of two ferrocyanide molecules formed per molecule of ascorbate added and resulted from the direct reaction between ascorbate and ferricyanide, generating DHA and ferrocyanide.

In figure 4 the dependence of the ferricyanide reduction on the concentrations of ascorbate and DHA is shown. In the case of ascorbate, the dose-response curve could not be extended to concentrations above 50 μM: the immediate reaction of ascorbate with ferricyanide caused excessive depletion of ferricyanide from the system at higher concentrations. The concentration range for DHA was limited to 1 mM, as higher concentrations of DHA interfered with the bathophenanthroline assay. The data in figure 4 show that the rate of ferricyanide reduction is dependent on the extracellular ascorbate or DHA concentration, and that it behaved in a saturable manner with a maximal rate of 0.41 nmol/10⁶ cells·min and an apparent K_m of 30 μM.

*p*CMBS, a sulfhydryl reagent, can be an effective inhibitor of the plasma membrane ferricyanide reductase (23, 24). *p*CMBS inhibited the reduction of ferricyanide in the absence of ascorbate, causing a 50% inhibition at 5.6 μM (Fig. 5). It also inhibited the ascorbate-stimulated reaction, although to a lesser extent, with a

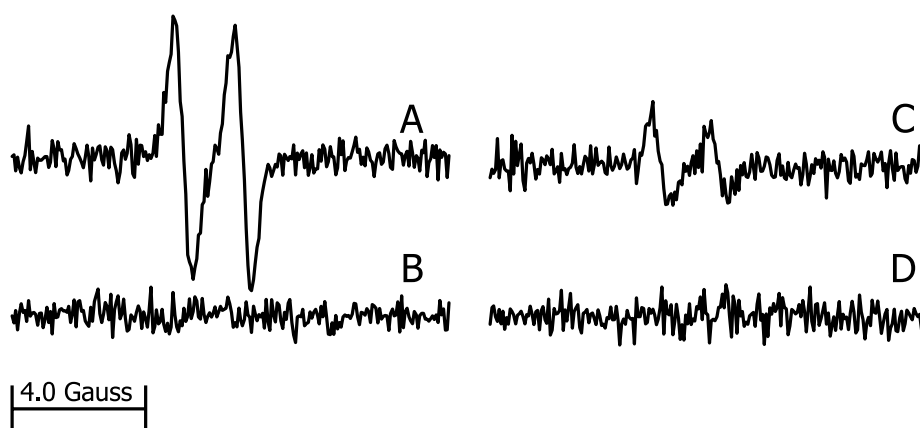


Figure 6: ESR spectra of the formation of the ascorbate free radical. (A) 25 μM ascorbate in Tris/NaCl; (B), as (A), but with 1 mM ferricyanide present; (C), as (A), but in the presence of $4 \cdot 10^6$ cells/ml; (D), as (B) but in the presence of $4 \cdot 10^6$ cells/ml.

50% inhibition estimated at 180 μM . This number was obtained through extrapolation of the data from figure 5. Concentrations above 100 μM *p*CMBS were not used, as they affected the intracellular accumulation of ascorbate.

Formation of the ascorbate free radical

The ascorbate free radical has been proposed as an intermediate in the accelerated reduction of ferricyanide by K562 cells (9). It is relatively stable and results from the one-electron oxidation of ascorbate. To test its involvement in the accelerated reduction of ferricyanide, the formation of the radical was measured under various experimental conditions (Fig. 6). The ascorbate free radical can readily be observed in a 25 μM ascorbate solution in buffer, with an ESR spectrum consisting of a doublet with hyperfine splitting $a^{\text{H}4} = 1.8 \text{ G}$ (Fig. 6A). Transition metal ions, which are always present in buffer solutions, mediate this formation of the ascorbate free radical (25). In the presence of 1 mM ferricyanide, no radical signal could be detected (Fig. 6B). The ascorbate free radical was also detected in the presence of HL60 cells, although the signal intensity was lower than in the absence of cells (Fig. 6C). This may be caused by a decreased ascorbate degradation, due to chelation of transition metal ions, or by an increased regeneration by an AFR-reductase. However, this was not further investigated, as it was considered to be outside the scope of this chapter. Again, in the presence of ferricyanide no radical signal could be detected (Fig. 6D).

Inhibitor	Ferricyanide Reduction		Ascorbate accumulation
	0 μ M ascorbate	25 μ M ascorbate	
None	38	206	667
20 mM D-glucose	28	78	149
20 mM 2-DOG	31	70	29
20 mM 6-DOG	23	94	96
Cytochalasin B			
Added before	39	50	<10
Added after	38	221	674

Table 1 : The role of the GLUT-1 transporter in ferricyanide reduction and ascorbate accumulation. Cells ($4 \cdot 10^6$ /ml) were incubated with the compounds listed for 15 min at 37 °C prior to the addition of 1 mM ferricyanide and 25 μ M ascorbate. In some experiments cytochalasin B was added 20 min after the addition of ferricyanide and ascorbate (i.e. added after). For cells incubated in the presence of ascorbate, its accumulation in HL60 was measured after 20 min of incubation and was expressed as pmol ascorbate/ 10^6 cells. Cells incubated in the absence of ascorbate did not contain any measurable ascorbate. Ferricyanide reduction (pmol ferrocyanide/ 10^6 cells·min) was determined as described in the Experimental Procedures.

Involvement of GLUT-1 transporter

Extracellular DHA is taken up by HL60 cells through a facilitative process, catalyzed by the GLUT-1 glucose transporter (11, 12). To test whether the activity of the GLUT-1 transporter was also involved in ascorbate or DHA-mediated ferricyanide reduction, the effect of several GLUT-1 substrates and inhibitors was studied. Table 1 shows that glucose and its analogs 2-deoxyglucose and 6-deoxyglucose strongly inhibited the ascorbate stimulated ferricyanide reduction, as well as the accumulation of ascorbate. Cytochalasin B, which inhibits GLUT-1 (11), was also an effective inhibitor. Ferricyanide reduction in the absence of ascorbate was hardly affected by these compounds, showing that they did not have an effect on the plasma membrane reductase.

Mechanism of enhanced ferricyanide reduction

Previous studies on erythrocytes suggest that ascorbate may serve as an intracellular electron donor for the plasma membrane reductase (26). The accumulation of ascorbate in HL60 leads to a similar concept, in which the reduction of ferricyanide and the oxidation of ascorbate are coupled. Comparison of the data in figures 2A and 3 shows that the amount of ferrocyanide formed exceeded the amount of

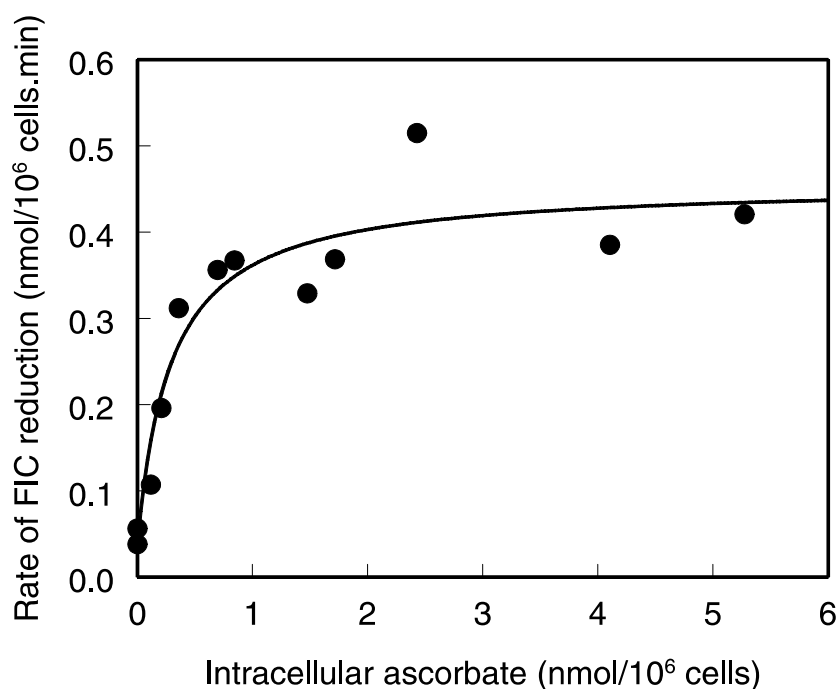


Figure 7: Correlation between the intracellular concentration of ascorbate and the rate of ferricyanide (FIC) reduction in HL60 cells. Data were obtained from uptake measurements and reductase assays in the same cell batch.

ascorbate in the cells: after 60 min, 12 nmol of ferricyanide was converted by 10^6 cells, while they contained 0.8 nmol ascorbate. As one molecule of ascorbate can reduce two molecules of ferricyanide, this amount of ascorbate accounted only for the reduction of 1.6 nmol ferricyanide. Thus, the presence of a recycling mechanism for ascorbate is required to explain the amount of ferricyanide reduced.

There are several ways by which intracellular ascorbate can reduce ferricyanide. It can either remain inside the cell and donate electrons to a redox system for ferricyanide reduction, or, alternatively, leave the cell to react with ferricyanide directly. In the latter case, DHA will be formed outside the cells, and has to re-enter the cell to be recycled to ascorbate. Thus, with a re-uptake step being essential, the stimulation of ferricyanide reduction should remain sensitive to GLUT-1 inhibitors throughout the entire incubation period. As shown in Table 1, this was not the case. Cytochalasin B inhibited only when added before the addition of ascorbate. When 5 μ M cytochalasin B was added 20 min after ascorbate, no inhibition was observed. This indicates that ascorbate remains in the cell and that intracellular ascorbate mediates the accelerated reduction of ferricyanide. Replotting the data in figures 2B and 4 results in figure 7, which shows the relation between the intracellular concentration of ascorbate and the rate of ferricyanide reduction.

α -Tocopherol added to medium	α -Tocopherol in cells	Ferricyanide reduction	
		0 μ M ascorbate	25 μ M ascorbate
μ M			
Grown with serum			
0	10	33	229
30	203	39	232
100	393	47	214
Grown serum-free			
0	0	29	242
30	202	45	292

Table 2: Effect of α -tocopherol on ferricyanide reduction. Ferricyanide reduction (pmol ferrocyanide/ 10^6 cells·min) and intracellular α -tocopherol levels (pmol/ 10^6 cells) were determined as described in the Experimental Procedures. Cells grown with serum were cultured under standard conditions, whereas cells without serum were cultured in a medium with serum replaced by transferrin, insulin and bovine serum albumin.

This rate correlated to the intracellular concentration in a saturable manner, resembling Michaelis-Menten kinetics. A fit of the data resulted in an apparent K_m of 0.29 nmol/ 10^6 cells, which corresponds to 0.55 mM (assuming a cell diameter of 10 μ m).

Glutathione is believed to be involved in the conversion of DHA to ascorbate inside the cell (27). Therefore, it was investigated whether glutathione depletion had an effect on the stimulation of ferricyanide reduction by ascorbate or DHA. Treatment of cells with BSO, an inhibitor of glutathione synthesis (17), caused a dramatic reduction in the cellular GSH/GSSG level, from 2.3 nmol/ 10^6 cells in the control to 0.07 nmol/ 10^6 cells. However, ferricyanide reduction rates were hardly affected by the depletion of glutathione. Also, ascorbate uptake measurements showed that intracellular ascorbate accumulation was not significantly affected by glutathione depletion (results not shown).

There have been reports suggesting a role for α -tocopherol in assisting the transport of electrons over the membrane (2, 28). Under standard culture conditions, cells have a very low α -tocopherol content, due to the low level of this compound in serum (29). In order to achieve α -tocopherol levels even lower than those in control cells, HL60 cells were cultured for several generations in absence of serum. Without this exogenous source of α -tocopherol, the α -tocopherol levels dropped below the detection limit of approximately 1 pmol/ 10^6 cells (Table 2).

Supplementation, on the other hand, caused at least a 20-fold increase in cellular α -tocopherol levels. Ascorbate-stimulated ferricyanide reduction was not significantly affected by supplementation or depletion of α -tocopherol.

Discussion

Ascorbate and DHA strongly increased the rate of ferricyanide reduction in HL60 cells, as has previously been found for K562 cells and erythrocytes (9, 10). For K562 cells, it was suggested that the stimulation of ferricyanide reduction by ascorbate was due to a plasma-membrane-localized AFR-reductase (9). However, it is highly unlikely that this mechanism explains our results. The reaction between ferricyanide and ascorbate is very fast, and the stoichiometry of the reaction amounted to 2 mol ferrocyanide formed per mol of ascorbate (Fig. 3). This indicates that the oxidation of ascorbate does not stop at the level of the ascorbate free radical, but that it continues to DHA. This was confirmed by ESR spectroscopy (Fig. 6). Addition of excess ferricyanide to a solution of ascorbate, as used in our experiments, resulted in the formation of DHA, not the ascorbate free radical. In the experiments with K562 cells a similar excess of ferricyanide was used, and it is therefore also unlikely that, in K562 cells, the enhanced reduction of ferricyanide by ascorbate is mediated by an AFR-reductase (9).

Another mechanism that has been proposed was based on studies on erythrocytes and involves the accumulation of ascorbate in cells, where it may serve as an intracellular electron donor for the plasma reductase (10). The data presented in this chapter also strongly suggest that intracellular accumulation of ascorbate in the cell is an essential part of the mechanism by which ascorbate or DHA has its effect on ferricyanide reduction. We found that only conditions that allowed the accumulation of ascorbate resulted in an accelerated reduction of ferricyanide (Fig. 2). In many cells, DHA is transported by a facilitative process, which is mediated by the GLUT-1 glucose transporter (11). Ascorbate, on the other hand, is not transported over the plasma membrane of HL60 cells. Our experiments corroborate this view, as ascorbate was accumulated in the cells only when DHA was present in the extracellular medium (Fig. 2). Several substrates and inhibitors of the GLUT-1 transporter blocked the accumulation of ascorbate, showing the involvement of this transport system (Table 1).

Intracellularly, DHA is reduced to ascorbate, as followed from control experiments using ^{14}C -labelled ascorbate. This conversion was also found in erythrocytes incubated with DHA (10). However, on incubation with ferricyanide erythrocytes

showed an efflux of DHA. As DHA is transported by the facilitative GLUT-1 transporter, it can be transported in both directions following a concentration gradient. Apparently, in erythrocytes, ferricyanide induced the formation of intracellular DHA from ascorbate, which could subsequently leave the cell. However, no significant efflux of DHA from HL60 cells could be observed, indicating the presence of an efficient regenerating system (Fig. 2). The major part of the DHA generated inside the cells was thus recycled to ascorbate, before it had a chance to be transported or hydrolyzed. Recently, Guaiquil et al. (12) reported that HL60 cells have an efficient ascorbate regenerating system, that does not require intracellular glutathione. The lack of effect of glutathione depletion on ascorbate accumulation found in our study corroborates this view. It was concluded that uptake of DHA into the cell, and its accumulation in the cell as ascorbate, are essential steps in the enhancement of ferricyanide reduction.

The stimulation of ferricyanide reduction by ascorbate was inhibited by GLUT-1 inhibitors or competing substrates (Table 1). This is in clear contrast with data recently obtained for erythrocytes, where it was found that inhibition of DHA transport by cytochalasin B did not impair the stimulation of ferricyanide reduction by ascorbate (13). This led to the conclusion that ascorbate was closely involved in the redox reaction, but that it acted both intra- and extracellularly. It was supposed that DHA could be regenerated to ascorbate independent of its cellular location. In HL60 cells this is clearly not the case. In these cells the GLUT-1 transporter is involved in stimulation of ferricyanide reduction by ascorbate and DHA.

The redox equivalents represented by intracellular ascorbate were exceeded many times (at least by a factor 8) by the total amount consumed by ferricyanide. This shows that ferricyanide reduction only lasts because ascorbate is continuously regenerated. Once ascorbate was accumulated in the cells, the addition of GLUT-1 inhibitors no longer had any effect on the rate of ferricyanide reduction (Table 1). Thus, the effect of ascorbate or DHA on ferricyanide reduction cannot be explained by an excretion and re-absorption of ascorbate from the medium. Instead, the data show that ascorbate has its effect inside the cell, serving either as a direct substrate for a redox system, or as an activator of such a system.

What redox system is responsible for the effect of ascorbate? It is possible that ascorbate-stimulated ferricyanide reduction uses the same system as the basal reduction of ferricyanide. However, when the effect of the inhibitor *p*CMBS on both the basal and the ascorbate-stimulated reduction is compared, this seems to be unlikely. The basal reduction is much more sensitive to inhibition by *p*CMBS than the accelerated reduction (Table 1). This indicates that the basal and the

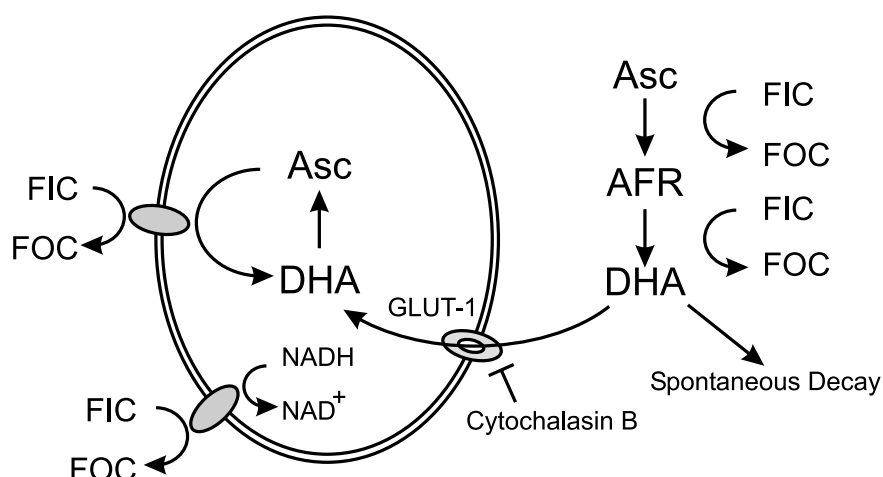


Figure 8: Model for the mechanism of the stimulation of ferricyanide reduction by ascorbate. FIC, ferricyanide; FOC, ferrocyanide; Asc, ascorbate.

accelerated reduction of ferricyanide are actually two separate processes, resulting in an additive reduction of extracellular ferricyanide. Summarizing our data, a model for the reduction of ferricyanide by HL60 cells is proposed in figure 8. At least two membrane redox systems are present in HL60 cells, both capable of reducing ferricyanide. The first system is the ferricyanide-reductase, which is thought to use NADH as its source for electrons. The second system is the one proposed in this chapter, and it relies on ascorbate for its reducing equivalents. It is interesting to note that under normal cell culture conditions there is only a limited supply of ascorbate to feed this system, since serum contains very low levels of ascorbate (30). Under physiological conditions, however, ascorbate is present at much higher concentrations. Therefore, this redox system may be a formidable addition to the cellular capability to counter oxidative processes at its surface, far exceeding the capacity of the NADH-dependent reductase.

The proteinaceous character of the basal ferricyanide-reductase seems well-established (24, 31-33), but the nature of the ascorbate-dependent system is not clear. It could involve either direct chemical reactions or a membrane-based enzyme (2, 28). There have been reports in the literature suggesting a non-enzymatic route of electron transport. In liposomes it was found that α -tocopherol, a natural anti-oxidant present in membrane lipids, mediated ferricyanide reduction by ascorbate without intervention of an enzyme system (2). Also, for erythrocytes, it was observed that α -tocopherol could augment ascorbate-induced ferricyanide reduction, but the involvement of a membrane-localized enzyme system could not be excluded (2). Conversely, the present results show that in HL60 cells the supplementation or depletion of α -tocopherol had no significant effect on the

efficacy of ascorbate in the stimulation of ferricyanide reduction (Table 2). Even in the total absence of α -tocopherol HL60 cells remain fully responsive to the addition of ascorbate. This unequivocally shows that α -tocopherol does not play a significant role in the stimulation of ferricyanide reduction by ascorbate and DHA in HL60 cells.

The intracellular level of ascorbate appeared to have a saturable dose-response relation with the observed rate of ferricyanide reduction, indicating that the reaction has Michaelis-Menten kinetics towards ascorbate. *p*CMBS inhibitor studies suggest the involvement of a protein, since this compound is well known to interfere with protein functions through its reactivity towards SH-groups. Thus, these experiments support the view that ascorbate-driven ferricyanide reduction proceeds through a redox system containing a proteinaceous component. However, further experiments will be needed to establish the exact nature of the system by which intracellular ascorbate can donate electrons to extracellular ferricyanide.

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