The ascorbate-driven reduction of extracellular ascorbate free radical by the erythrocyte is an electrogenic process

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Abstract Erythrocytes can reduce extracellular ascorbate free radicals by a plasma membrane redox system using intracellular ascorbate as an electron donor. In order to test whether the redox system has electrogenic properties, we studied the effect of ascorbate free radical reduction on the membrane potential of the cells using the fluorescent dye 3,3′-dipropylthiadicarbocyanine iodide. It was found that the erythrocyte membrane depolarized when ascorbate free radicals were reduced. Also, the activity of the redox system proved to be susceptible to changes in the membrane potential. Hyperpolarized cells could reduce ascorbate free radical at a higher rate than depolarized cells. These results show that the ascorbate-driven reduction of extracellular ascorbate free radicals is an electrogenic process, indicating that vectorial electron transport is involved in the reduction of extracellular ascorbate free radical.

Key words: Ascorbate; Ascorbate free radical; Plasma membrane; Membrane potential; Redox system

1. Introduction

Ascorbate, or vitamin C, is involved in the protection of an organism against a variety of oxidative agents [1,2]. However, the reaction with an oxidant consumes ascorbate, thus leading to loss of the vitamin. Oxidation of ascorbate usually takes place in two one-electron steps, the first of which results in the ascorbate free radical (AFR) [3]. AFR can be oxidized further, producing dehydroascorbic acid (DHA). Also, two molecules of AFR can disproportionate, forming one DHA and one ascorbate molecule. The ring structure of DHA is easily opened by an irreversible hydrolysis reaction [4]. Hence, failure to quickly reduce oxidation products of ascorbate will lead to loss of the vitamin. To prevent ascorbate depletion, DHA and AFR can be reduced by a number of systems, most of which are located in the cytoplasm of the cell. The conversions are mainly enzymatic, by e.g. glutaredoxin, thioredoxin reductase, or AFR reductases, but a chemical reduction by glutathione alone has also been described [5–9]. When an oxidation occurs extracellularly, the product DHA can be transported into the cell for reduction. For erythrocytes, it has also been described that AFR and DHA can be reduced extracellularly by redox enzymes in the plasma membrane, which are thought to use intracellular NADH as a source of reducing equivalents [10,11]. Recently, we found evidence for an alternative pathway for the reduction of extracellular AFR in the erythrocyte (Fig. 1) [12]. Not NADH, but intracellular ascorbate provided the reducing equivalents for this reaction, which may involve a transmembrane redox enzyme. The reaction has high similarity to a redox process in the adrenal chromaffin granules. In these granules, a cytochrome b_{561} is involved in the transmembrane reduction of AFR by ascorbate [13–15]. Earlier, we investigated whether this cytochrome was also expressed in the erythrocyte, but it was found that this was not the case [16]. It is possible that another, similar, erythrocyte redox system is responsible for the reduction of AFR. On the other hand, it has also been suggested that electrons can be transported over the membrane by small lipid soluble molecules like α-tocopherol and coenzyme Q [17–19]. Thus, the mechanism of the redox system remains unresolved.

This paper further investigates the mechanism of the reduction of extracellular AFR by intracellular ascorbate. Previous data indicated that an electron is abstracted from intracellular ascorbate, and transported over the membrane. In principle, such a movement of charge is electrogenic, and could change the potential of the plasma membrane. Other transmembrane redox processes were indeed shown to be electrogenic. In cells and vesicles from different cell types and species, the reduction of extracellular substrates like ferricyanide was found to affect the membrane potential, resulting in depolarization [20–24].

To test whether the reduction of AFR by the plasma membrane redox system of erythrocytes has electrogenic properties, changes in the membrane potential were studied using a potential sensitive fluorescent carbocyanine probe. Moreover, the effect of different membrane potentials on the reductase activity was determined. It is concluded that the ascorbate-driven plasma membrane redox system indeed catalyzes electrogenic AFR reduction, and that it is not likely that small electron carriers are involved in the transfer of electrons over the plasma membrane.

2. Materials and methods

Chemicals were from Sigma (Zwijndrecht, The Netherlands) unless specified otherwise. Ascorbate oxidase (EC 1.10.3.3) was purchased as sticks containing 17 U of enzyme (Roche Diagnostics, Almere, The Netherlands), and was freshly dissolved before use by gentle mixing for 30 min. Erythrocytes were obtained from 1-day old citrate-anticoagulated blood, collected from healthy human volunteers by the

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Abbreviations: AFR, ascorbate free radical; DHA, dehydroascorbic acid; $DiSC_3(5)$, 3,3′-dipropylthiadicarbocyanine iodide; ESR, electron spin resonance; TEMPO, 2,2,6,6-tetramethylpiperidine-*N*-oxyl

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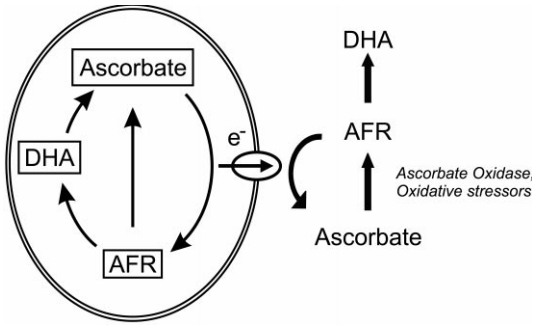

Fig. 1. Model for the ascorbate-dependent reduction of AFR. Intracellular ascorbate can donate an electron to a system in the plasma membrane, which subsequently reduces extracellular AFR. Thus, the net oxidation of extracellular ascorbate is decreased. Intracellular AFR is quickly regenerated to ascorbate. NADH can also serve as an intracellular electron donor for the reduction of extracellular AFR, but was omitted from this scheme.

Bloodbank Leiden/Haaglanden (Leiden, The Netherlands). The cells were washed three times in 20 mM Tris, 150 mM NaCl pH 7.4 (Tris/NaCl). The buffy coat of white cells was removed carefully with each wash.

Erythrocytes were loaded with ascorbate by resuspending cells to a hematocrit of 20% in Tris/NaCl with 500 μM DHA, and 2.5 mM adenosine as an energy-source [25]. Control erythrocytes were treated similarly, but without DHA. After 30 min of incubation at room temperature, the erythrocytes were washed three times with Tris/NaCl, and used within 1 h for subsequent experiments. As described before, ascorbate-loaded erythrocytes contained 1 mM ascorbate, while control erythrocytes contained up to 50 μM endogenous ascorbate [12]. All experiments were performed in Tris/NaCl, or, where indicated, in a similar buffer where a part of the NaCl was replaced by KCl.

Membrane potentials were determined using the potential sensitive dye 3,3′-dipropylthiadicarbocyanine iodide ($DiSC_3(5)$, Molecular Probes, Leiden, The Netherlands) [26]. 1 μM of dye was added to a 1% suspension of erythrocytes in a stirred plastic cuvette, and fluorescence was monitored using a Perkin-Elmer LS-50B fluorimeter and FL Winlab 3.0 software (Perkin-Elmer Benelux, Oosterhout, The Netherlands). The excitation wavelength was set at 640 nm, and emission at 670 nm, both using a 10-nm slit.

The concentration of AFR was determined using electron spin resonance (ESR) spectroscopy. Spectra were obtained using a JEOL-RE2X spectrometer operating at 9.36 GHz with a 100-kHz modulation frequency, equipped with a TM_{110} cavity. Immediately after mixing, the samples were transferred to the quartz flat cell by means of a rapid sampling device, and a spectrum was recorded using a 2-min scan time. The area under the ESR absorption curve, as determined by double integration, is proportional to the amount of paramagnetic species in the sample, and this can be used as a quantitative assay. The concentration of AFR was determined by double integration of the ESR spectra, using 2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO) as a standard. The spectra were obtained with the same instrument settings as used for collecting the experimental spectra, except for receiver gain. ESR spectrometer setting were as follows: microwave power, 40 mW; modulation amplitude, 0.63 G; time constant, 0.03 s; scan time, 2 min; scan width, 25 G.

The reduction of AFR was measured indirectly by monitoring the oxidation of 100 μM ascorbate (Aldrich, Zwijndrecht, The Netherlands) in a 10% suspension of erythrocytes [12]. AFR was generated by oxidizing 100 μM ascorbate with 10 mU/ml of ascorbate oxidase. At 0 and 15 min, a sample of the suspension was centrifuged, and the amount of ascorbate remaining in the supernatant was measured immediately. The rate of ascorbate oxidation in the sample is determined from the difference in concentration between 0 and 15 min. Ascorbate concentrations were determined by measuring the absorption at 265 nm in a Beckman DU-65 spectrophotometer (extinction coefficient = 14 500 M^{-1} cm^{-1}).

All experiments were performed at least three times, and error bars represent the standard deviation where applicable.

3. Results and discussion

3.1. The effect of AFR on the membrane potential

Addition of 1 μM $DiSC_3(5)$ to ascorbate-loaded or control erythrocytes induced a sharp increase in fluorescence, followed by equilibration at a lower level after the dye partitioned between the medium and the cells (Fig. 2). Subsequently, 100 μM ascorbate and 42.5 mU/ml ascorbate oxidase were added in order to generate ascorbate radicals in the suspension. As shown in Fig. 2, AFR had no measurable effect on the fluorescence in the presence of control erythrocytes. However, in the presence of ascorbate-loaded erythrocytes the fluorescence increased, indicating a depolarization of the membrane. The addition of 100 μM ascorbate or 42.5 mU/ml ascorbate oxidase alone to a suspension of ascorbate-loaded erythrocytes did not affect the fluorescence. The membrane potential was only affected in presence of both agents, irrespective of the order in which they were added (not shown). Addition of DHA instead of ascorbate and ascorbate oxidase did not alter the fluorescence. Also, control experiments showed that none of the additions affected $DiSC_3(5)$ fluorescence in the absence of cells. The depolarization of the erythrocytes therefore depended on the formation of AFR in the suspension.

To further study the effect of AFR on the membrane potential, different amounts of AFR were generated in a suspension of ascorbate-loaded erythrocytes. This was done by varying the amount of ascorbate oxidase in a suspension containing 100 μM ascorbate. Under similar conditions, the corresponding AFR concentrations were measured by ESR spectroscopy. As shown in Fig. 3, the extent of erythrocyte depolarization, expressed as the shift in $DiSC_3(5)$ fluorescence upon addition of AFR, depended on the concentration of extracellular AFR in the suspension.

Depolarization of ascorbate-loaded erythrocytes in the presence of extracellular AFR shows that the reduction of AFR must indeed be an electrogenic process. The transfer of charge over the membrane during the reduction of AFR agrees with the model of a transmembrane redox reaction with ascorbate as an intracellular electron donor.

Untreated erythrocytes can also reduce extracellular AFR,

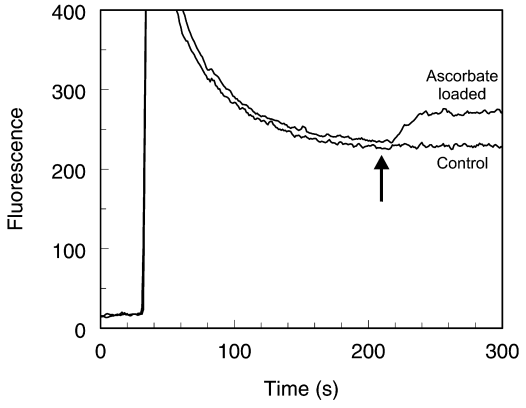

Fig. 2. Depolarization of ascorbate-loaded erythrocytes in the presence of AFR. 1 μM $DiSC_3(5)$ was added to a 1% suspension of either control or ascorbate-loaded erythrocytes at 30 s, and 100 μM ascorbate and 42.5 mU/ml ascorbate oxidase after equilibration of the dye at 210 s. After equilibration of the dye, an increase in fluorescence indicates a depolarization of the membrane.

albeit at a lower rate than ascorbate-loaded cells [10,12]. Nevertheless, no changes in the membrane potential were observed upon exposure of control erythrocytes to AFR (Fig. 2). We earlier showed that these cells can use NADH and endogenous ascorbate as electron donors for the reduction [12]. Endogenous ascorbate is typically present in the range of 20–50 μM, while ascorbate-loaded erythrocytes contained 1 mM ascorbate [27]. The most likely explanation for the lack of change in the membrane potential of control erythrocytes would therefore be that the rate of charge transfer is too low to produce a detectable effect.

3.2. The effect of membrane potential on AFR reduction

It is conceivable that the membrane potential of a cell affects the transport of electrons over the membrane. In order to test whether this was the case, we studied the reductase activity by measuring the ascorbate stabilizing effect of cells [12]. In this assay, ascorbate is degraded to AFR by ascorbate oxidase. The reduction by the cells of AFR to ascorbate decreases the apparent rate of this reaction. For clarity, the difference between the ascorbate oxidation rate in each sample and the rate in buffer alone (3.68 μM/min) was defined as the apparent rate of AFR reduction. Several membrane potentials were established in erythrocytes by suspending them in buffers with different concentrations of potassium and 1 μM valinomycin. It was found that the rate of AFR reduction was dependent on the membrane potential of the erythrocytes. Hyperpolarization of the erythrocytes increased AFR reduction, whereas it was decreased by a depolarization of the cells (Fig. 4). The clear correlation of the reduction rate with the membrane potential confirms the view that electrogenic transmembrane electron transport is involved.

The data presented in this paper show that the reduction of AFR by erythrocytes causes depolarization, and that depolarization causes decreased reduction of AFR by the ascorbate-driven redox system. This could imply that this reduction of AFR is auto-inhibitory. However, it must be noted that the depolarization in our experiments required relatively high concentrations of AFR. The levels of the radical used in Fig. 4 were lower, and the levels in a physiological setting will probably be even less, preventing significant changes in the membrane potential. It is therefore unlikely that a change in membrane potential is a limiting factor in the physiological reduction of AFR.

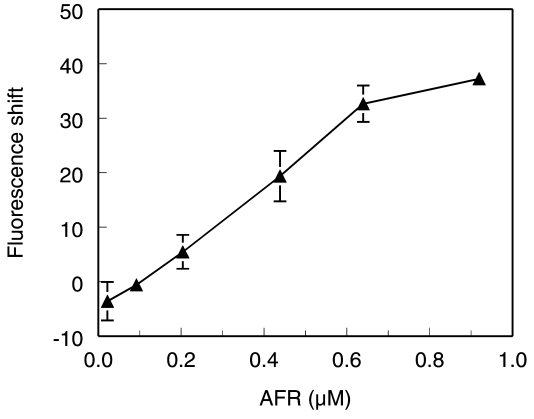

Fig. 3. Correlation between erythrocyte depolarization and the concentration of AFR. 1 μM $DiSC_3(5)$ was added to a 1% suspension of ascorbate-loaded erythrocytes. After equilibration, 0, 1, 5, 20, 42.5 or 100 mU/ml ascorbate oxidase and 100 μM ascorbate were added to the suspension, and the subsequent increase in fluorescence was recorded. The data are the average of three experiments, and error bars represent the standard deviation. The corresponding AFR concentrations were established by ESR spectroscopy.

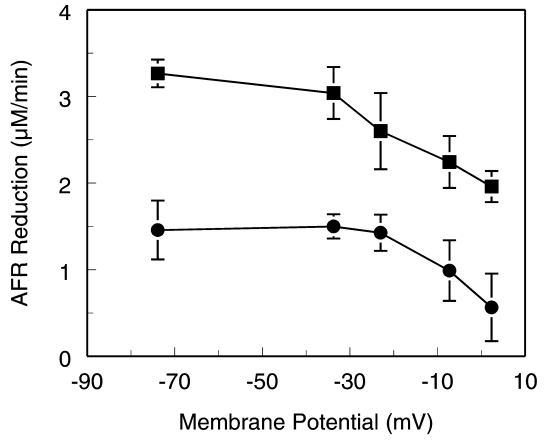

Fig. 4. Effect of erythrocyte membrane potentials on the reduction of AFR. 100 μM ascorbate and 10 mU/ml ascorbate oxidase were added to a 10% suspension of control (●) or ascorbate-loaded (■) erythrocytes. The erythrocytes were suspended in buffers containing 0, 25, 50, 100 or 150 mM potassium and 1 μM valinomycin to establish different membrane potentials. Subsequently, the apparent rate of AFR reduction was established by measuring the rate of ascorbate oxidation, as described in the text. The data are the average of three experiments, and error bars represent the standard deviation. The membrane potentials in the figure were calculated using $V_m = 58\ \log((\alpha[K]_o+[Cl]_i)/(\alpha[K]_i+[Cl]_o))$ [28]. Constants were used as follows: $\alpha = 20$ (the ratio of the permeabilities of K and Cl in presence of valinomycin); $[K]_i = 136$ mM (intracellular K); $[Cl]_i = 105$ mM (intracellular Cl); $[Cl]_o = 150$ mM (extracellular Cl) [29]. Normal resting erythrocytes have a membrane potential of about −9 mV [29].

3.3. Conclusion

The data in this study provide evidence for the transmembrane nature of the ascorbate-dependent reduction of extracellular AFR by the erythrocyte. The model for the reduction of extracellular AFR that was suggested by earlier data now seems firmly established. Intracellular ascorbate donates a single electron to a system in the plasma membrane, which passes it on to reduce extracellular AFR (Fig. 1). The electrogenic nature of the electron transfer provides information on the nature of the system in the plasma membrane. It has been suggested that small lipid soluble molecules like α-tocopherol and coenzyme Q can act as electron shuttles in the plasma membrane. Thus, they could transfer electrons from e.g. intracellular ascorbate to extracellular AFR. However, these electron shuttles bind a proton when accepting an electron, which means that a proton is transported together with the electron, and that no net charge crosses the membrane. However, our data show that the reduction of AFR is electrogenic, which implies that α-tocopherol or coenzyme Q do not play a major role in this process. Instead, it is more likely that one or more proteins in the erythrocyte membrane are involved in the electron transfer process.

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