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THE HYPOXIC STRESS ON ERYTHROCYTES ASSOCIATED WITH SUPEROXIDE FORMATION

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Superoxide is produced during the autoxidation of hemoglobin. Autoxidation of hemoglobin is, however, facilitated under hypoxic conditions where hemoglobin is only partially oxygenated.

We have recently found that the erythrocyte superoxide dismutase does not fully react with the additional superoxide produced under hypoxic conditions. A leakage of superoxide from the erythrocyte is thus detected, resulting in a potential source for oxyradical damage to tissues.

Detailed studies on intact erythrocytes as a function of oxygen pressure have now been performed. These studies further delineate the hypoxic stress on erythrocytes and the mechanism for the leakage of superoxide. By centrifugation of samples under various oxygen pressures it was possible to show an enhanced rate of lysis at reduced oxygen pressures with a maximum rate in the region of 25 mm Hg. At much lower pressures where the hemoglobin is mostly deoxygenated the rate of lysis was dramatically decreased with almost no lysis detected even after three days. Lysis is shown to be associated with superoxide membrane damage. The formation of superoxide which does not react with endogenous SOD reaches a maximum value at much lower pressures where most of the hemoglobin is deoxygenated. It is suggested that the leakage at low pressure is associated with the formation of superoxide by oxidation of hemoglobin associated with the membrane.

KEY WORDS: erythrocytes, superoxide, lysis, hypoxia, oxygen, oxidation.

INTRODUCTION

The oxidative stress and potential damage associated with oxyradical formation has received considerable interest in recent years.[1,2] The concentrations of these species are in general expected to increase as a function of the oxygen pressure. However, recent results suggest a hypoxic stress associated with oxyradicals even at low oxygen pressures. Elevated lipid peroxidation has, thus, been reported for rats kept under chronic hypoxia for two weeks.[3] Furthermore, it has been found that tissue and cells incubated at low oxygen pressures are more sensitive to oxyradical damage during the reoxygenation process.[4,5]

We have previously proposed as a possible source for hypoxic stress the enhanced rate of oxidation of hemoglobin at reduced oxygen pressures.[6,7] This phenomenon was attributed to the relative instability of partially oxygenated hemoglobin and the resultant formation of superoxide. Enzymatic processes in erythrocytes dramatically lower the level of oxidized hemoglobin. Thus even during incubation at reduced oxygen pressures for several days only a minor fraction of the hemoglobin is oxidized.

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In this paper we demonstrate enhanced lysis and formation of superoxide under these conditions. A hypoxic stress is thus observed even though the level of oxidized hemoglobin remains low.

MATERIALS AND METHODS

Erythrocyte Studies

Erythrocytes were obtained from fresh human blood washed in isotonic media to remove plasma and buffy coat. Experiments were performed on dilute suspensions of cells (~0.6%) in phosphate buffered saline (PBS) pH 7.4 with 0.1 mM ethylenediamine-tetraacetic acid (EDTA) or diethylenetriamine-pentaacetic acid (DETAPAC). This dilution was necessary to perform optical studies in a 1 cm pathlength optical cell.

The oxygen pressure in these cells was adjusted by evacuating a ballast of known volume into which the oxygen in the spectroscopic cell could be diluted. After the pO_2 was adjusted a specially designed ground glass joint was rotated to seal the cell. Spectroscopic analysis and lysis were determined directly on the cell suspension in these sealed cells. Spectra were obtained from 640–490 nm on a Perkin Elmer lamda 6 with a scattering accessory which made it possible to obtain spectra from intact cells. Spectra were analyzed in terms of a linear sum of individual spectral components. Lysis was determined by using a specially designed centrifuge which made it possible to spin down the cells at specified times and to separately analyze the supernatants for hemoglobin content. Spectroscopic analysis on the cells was obtained by subtracting the spectrum of the supernatant from that of the total cell suspension.

Cytochrome C

Cytochrome *C* (Type VI) from Sigma (St. Louis) was succinylated.[8] Oxidized cytochrome *C* was added to the erythrocyte suspension. Reduction of cytochrome *C* in these suspensions was determined by the use of component spectra for oxidized and reduced cytochrome *C* in the fitting procedure.

Superoxide dismutase (SOD) obtained from Sigma (St. Louis) was added to the rythrocyte suspension (500 U/ml) to delineate the fraction of cytochrome *C* reduction which could be inhibited by SOD. This fraction is frequently attributed to superoxide reduction.[9,10] However, hemoglobin is also able to slowly reduce cytrochrome *C*[11] and the contribution of hemoglobin to the observed cytochrome *C* reduction needs to be considered.

Nitroblue Tetrazolium (NBT)

The reduction of NBT is another method frequently used to measure superoxide.[12] In our studies on erythrocyte reduction of NBT we have added NBT (obtained from Sigma, St. Louis) to the erythrocyte suspension. Since the reduced NBT is relatively insoluble in water[13] we found that a quantitative measure of the reduction requires extraction into an organic solvent. Therefore, at the end of the erythrocyte incubation studies the spectroscopic cells were opened and 2 ml of the cell suspension were mixed with 2 ml of dimethyl phthalate (obtained from Eastman Kodak, Rochester) and vortexed for 1 min. These samples were centrifuged to separate the phases and optical

spectra from 640 nm to 490 nm were obtained on the organic phase. A solution of potassium superoxide (KO_2) dissolved in dry dimethyl sulfoxide was used to calibrate the reduction of NBT enabling us to quantitate the superoxide reacted with NBT. By performing these calibrations with and without erythroyctes in both an O_2 and N_2 atmosphere,[12] a possible enhancement of the reaction in the absence of oxygen was ruled out.

The spectrum of the extracted solution was measured after the addition of a series of KO_2 aliquots to NBT both in the absence and presence of erythrocytes. NBT without added KO_2 gave no absorption in the extracted solution. From the maximum absorption obtained in the absence of erythrocytes it was possible to determine the spectrum of the fully reduced NBT necessary to quantitate the superoxide reaction. An absorption spectrum was obtained when an NBT solution containing erythrocytes, oxyhemoglobin or carboxyhemoglobin was extracted into dimethyl phthalate even without added KO_2. However, as shown in Figure 1, this spectrum was clearly distinct from that which is obtained when superoxide is added to NBT. This spectral component is attributed to a non-speroxide reduction of NBT.[14] Fitting the data from incubated erythrocytes, required the use of both of these components.

RESULTS

Figure 2 shows that the hypoxic stress associated with the incubation under reduced oxygen pressures results in both enhanced lysis and the excessive formation of superoxide which can be detected even though the erythrocytes contain relatively high levels of SOD.[15]

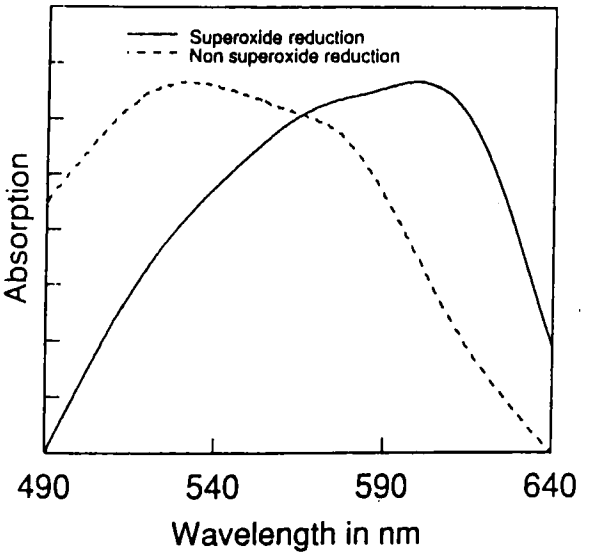

FIGURE 1 The absorption spectra of the dimethyl phthalate extracts of NBT solutions. (——) 2×10^{-5} M NBT dissolved in PBS (no hemoglobin) with an excess of KO_2 added before extraction; (– – –), same extraction in the presence of erythrocytes and no added KO_2. At a concentration of 2×10^{-5} M NBT and 1×10^{-4} M heme the intensity of the superoxide absorption relative to that of the non superoxide absorption is 7:1.

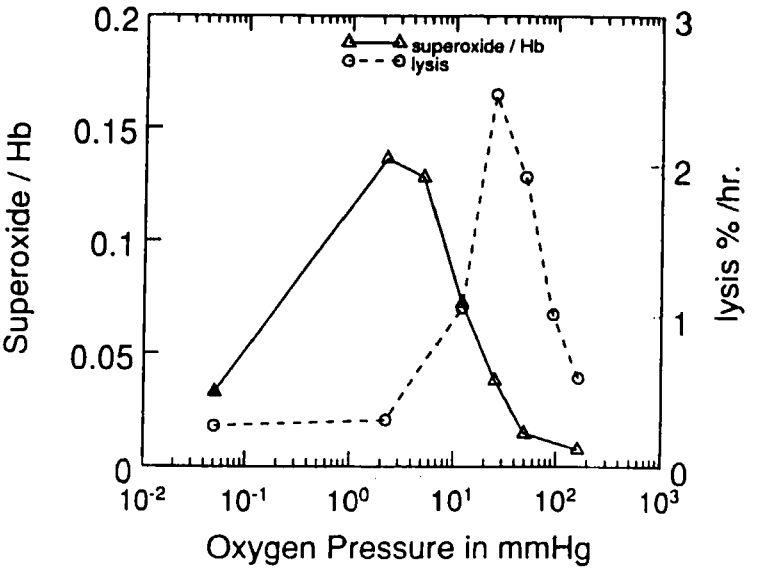

FIGURE 2 The oxygen dependence of erythrocyte lysis (– – o – –) and superoxide formation after 24 hours (-▵-). Superoxide formation is measured by NBT reduction. Corrections are made for non superoxide reduction. The solid point (▲) was obtained by using CO to remove the residual oxygen bound to hemoglobin at low oxygen pressures.

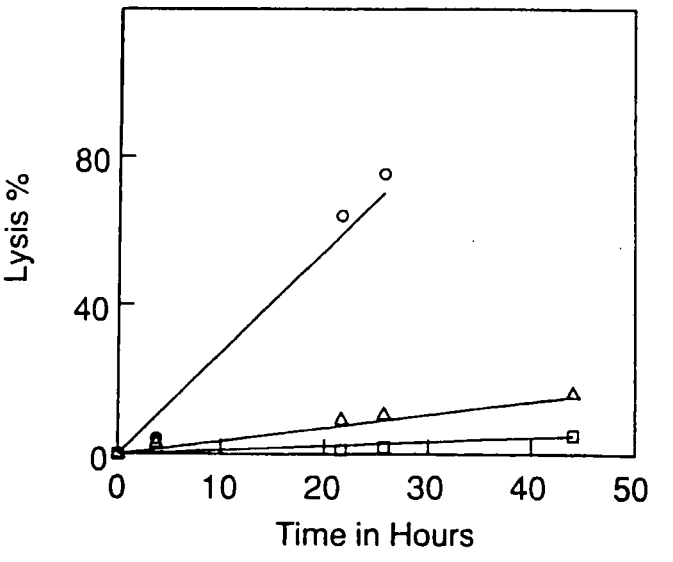

FIGURE 3 Time dependence of erythrocyte lysis at an oxygen pressure of 48.5 mm Hg. (o), erythrocytes in PBS, pH 7.4, 1 mm EDTA; regression line corresponds to 1.90%/hr lysis. (▵), erythrocytes in PBS, pH 7.4, 1 mm EDTA + 500 U/ml SOD; regression line corresponds to 0.35%/hr lysis. (□), erythrocytes in PBS, pH 7.4, 1 mm EDTA, 0.1 mM NBT. Regression line corresponds to 0.11%/hr lysis.

Lysis

Lysis is enhanced as the pO_2 is reduced with a maximum in the rate of lysis in the range of 25 mm Hg with approximately 30% of the hemoglobin deoxygenated. The enhancement in the rate of lysis at the early stages of hemoglobin deoxygenation is consistent with the results on hemoglobin, which indicate a maximum rate of oxidation and formation of superoxide in the range of 40% deoxygenated hemoglobin.[7]

This enhanced rate of lysis is shown in Figure 3 to be due to superoxide damage. Thus, SOD added at a concentration of 500 U/ml to the cell suspension produces a 6-fold decrease in the rate of lysis. The effect of SOD added to the outside of the cell suggests that a major factor in the lysis is superoxide damage from outside the cell. This superoxide could originate either outside the cell or from superoxide formed in the cell which leaks through the anion channel[16,17] and damages the membrane from the outside.

The greater protection from lysis produced by NBT (Figure 3) can be attributed to the ability of NBT to penetrate the membrane and prevent lysis by reacting with superoxide even before it leaks out of the erythrocyte. These results may be attributed to a specific form of membrane damage found with superoxide.[18] A role for superoxide in erythrocyte lysis has previously been reported under other conditions.[19,20]

Generation of Superoxide

The excessive superoxide generated by erythrocytes under hypoxic conditions has been measured both by the reduction of succinylated cytochrome *C* as well as by the reduction of nitroblue tetrazolium (NBT). The results obtained for the reduction of cytochrome *C* are shown in Table I. Measuring the cytochrome *C* reduction non-inhibitable by SOD, it is found that after 25 hour incubation the maximum reduction coincides with the maximum rate of lysis.

With the absence of added SOD to inhibit lysis, (see Figure 3) an appreciably greater concentration of hemoglobin is in contact with the cytochrome *C*. Shown in Table I are the increased hemoglobin concentrations found in the supernatant when SOD is not added together with the cytochrome *C*. In all cases the increased hemoglobin when SOD is not added is greater than the increased cytochrome *C* reduction when SOD is not added. Furthermore larger increases in hemoglobin concentration are observed at the higher pressures where the reduction and the lysis are maximal.

Since oxyhemoglobin can directly reduce cytochrome *C* at a slow rate[11] (we have

TABLE I
Reduction[a] of Cytochrome *C*[b] by Erythrocytes

Pressure (mm Hg)	Δ Reduction[c] (μM)	Δ Hemoglobin[d] (μM)
158.9	4.7	44.7
48.5	15.1	55.7
2.21	6.5	10.4
0.05	4.1	5.61

[a] After 24 hour incubation at ambient temperature
[b] Cytochrome *C* was modified by succinylation of lysine ε-amino groups
[c] A correction is made for the reduction in the presence of superoxide dismutase (500 U/ml).
[d] The total hemoglobin due to lysis in contact with cytochrome *C*. A correction is made for the lysis that occurs in the presence of superoxide dismutase (500 U/ml).

found 20% of the cytochrome *C* reduced after 3 hours with equal concentrations of cytochrome *C* and oxyhemoglobin) it is not clear to what extent the cytochrome *C* reduction can be attributed to superoxide. This uncertainty exists even after correcting for the reduction in the presence of SOD.

Using NBT reduction, it was possible to eliminate any contribution arising from direct interactions with hemoglobin. As shown in Figure 1, the spectrum obtained by the addition of superoxide is clearly distinct from the spectrum obtained for hemoglobin or erythrocytes without superoxide. By fitting the spectra obtained for the dimethyl phthalate extraction with both components (Figure 1), the contribution of superoxide reduction is determined, and the contribution of other reduction processes[14] is eliminated.

Table II shows the effect of SOD on the reduction of NBT. At atmospheric pressure no significant superoxide component is observed with or without SOD. The superoxide component becomes appreciable at 5 mm Hg with the reduction of NBT decreased by 60% in the presence of SOD. The non-superoxide component is appreciable at both O_2 pressures with a much smaller inhibition by SOD.

In an attempt to compare the cytochrome *C* reaction with the NBT reaction, parallel studies on the same blood sample were performed using NBT and cytochrome *C*. At the lowest pressures where the excessive lysis becomes negligible (see Table I), the superoxide reduction measured by NBT and cytochrome *C* are very similar, while at the higher pressures where lysis is appreciable, much less NBT reduction is observed and the higher cytochrome *C* results are presumably due to hemoglobin reduction of cytochrome *C*.

The NBT reaction is thus a more reliable measure of superoxide formation in the erythrocyte system. In Figure 2 we have therefore used the NBT-determined superoxide component (see Figure 1) as an indication of superoxide formation. The low levels of superoxide/Hb (0.02–0.08) found in the pressure range where lysis is dominant most reflect the superoxide which produces the lysis. The reaction of NBT with this superoxide is thus responsible for the inhibition of lysis (Figure 3). As seen in Figure 2, the dominant superoxide formation occurs at lower O_2 pressures where the data indicate that not more than a few percent of the hemoglobin is oxygenated.

We actually found that appreciable inhibition of the superoxide formation could not be obtained by diluting the oxygen from the hemoglobin into a ballast. It was therefore necessary to ascertain that the NBT reduction was actually the result of residual oxygen bound to hemoglobin. For this purpose we used CO to displace the remaining oxygen after the oxygen pressure had been reduced by dilution into a ballast. Thus results shown by the solid point in Figure 2 demonstrate that the

TABLE II
Effect of Superoxide Dismutase[a] on the reduction of Nitroblue Tetrazolium (NBT)[b]

Pressure	SOD	Superoxide reaction	Non-Superoxide reaction
atmos	–	N.S.	3.7×10^{-6} M
atmos	+	N.S.	3.5×10^{-6} M
5 mm Hg	–	2.5×10^{-6} M	4.6×10^{-6}
5 mm Hg	+	1.0×10^{-6} M	3.6×10^{-6}

[a] Superoxide dismutase was added to the supernatant at a concentration of 500 U/ml.
[b] The concentration of reduced NBT is based on an extinction coefficient for the non-superoxide product equal to that of the superoxide product.

superoxide formation measured by NBT is actually associated with oxygen bound to hemoglobin.

DISCUSSION

The enhanced formation of superoxide at low oxygen pressures coincides with a dramatic drop in the rate of lysis (Figure 2). Considering the maximal rates of hemoglobin oxidation in the middle of the oxygenation curve[7] the determination of more superoxide at low pressures can not be due to a greater rate of formation, but must be due to the inaccessibility of the superoxide formed at low pressures to the intracellular SOD.

The existence of such a fraction of superoxide is also supported by the results of Table II which show that at 5 mm Hg only 60% of the NBT reaction is inhibited by exogenous SOD. This is somewhat less than the usually reported 70–90% inhibition of NBT reduction by SOD.[14] The finding that lysis is inhibited to a greater extent by NBT than SOD (Figure 3) actually suggests that a fraction of superoxide that does not react with endogenous SOD is present even at higher pressures.

This phenomenon can be attributed to interaction of hemoglobin with the membrane Band 3 on the cytoplasmic surface of the membrane.[21,22] Recent studies indicate that deoxyhemoglobin binds to Band 3 more readily than oxyhemoglobin.[23] Therefore, the monoliganded hemoglobin present at low oxygen pressures is presumably associated with the membrane. Superoxide formed from hemoglobin associated with Band 3 is located near the anion channel and can leak out of th erythrocyte without reacting with intracellular SOD.[16]

At higher oxygen pressure with reduced membrane interaction, a greater portion of the superoxide formed by oxidation reacts with SOD. However, a small amount reaches the membrane resulting in membrane damage and cell lysis. The coincidence of the drop in lysis with the rise in superoxide (Figure 2) may also suggest a stabilizing effect on the membrane associated with hemoglobin binding.

CONCLUSION

A hypoxic stress on erythrocytes is indicated which results in greater lysis at intermediate oxygen pressures when deoxygenation of hemoglobin is beginning to take place. However, at low pressures the membrane interactions with the hemoglobin prevent lysis but result in the release of superoxide from the cells. This superoxide provides a potential hypoxic stress to tissues and organs.

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