Binding of haemoglobin to the red-cell membrane in the presence of copper chloride

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(Received 3 November 1983/Accepted 27 January 1984)

Methaemoglobin may be an important factor for initiation and development of lipid peroxidation in Cu(II)-treated red blood cells. It seems likely that the initiation of peroxidation by methaemoglobin is only possible if direct contact between haemoglobin molecule and the cell membrane is realized. In view of this, the binding of haemoglobin to the red-blood-cell membrane in the presence of CuCl₂ was studied. It was found that the haemoglobin quenching of the fluorescence of 12-(9-anthroyl)stearic acid-labelled red-blood-cell membranes greatly increases in the presence of CuCl₂. This effect is relatively independent of pH and the ionic strength of the medium, indicating that in this case the binding of haemoglobin is not electrostatic in nature. The haemoglobin quenching of the fluorescence of the inside-out and the right-side-out resealed ghosts were almost the same in the presence of CuCl₂. This result suggests that, in the presence of ionic copper, both surfaces of the membrane possess approximately equal amounts of sites for the binding of haemoglobin.

The mechanism of the copper haemolytic action is essentially unknown, but the suggestion has been made that processes involving radicals and mediated by oxygen are involved (Hochstein *et al.*, 1978). In support of this hypothesis, in our previous study (Ribarov & Benov, 1981) it was found that CuCl₂-induced haemolysis *in vitro* is preceded by the development of lipid peroxidation. Kumar *et al.* (1978) and Ribarov & Bochev (1984) have shown generation of O₂^{•-} as a result of CuCl₂-RBC membrane interaction. Furthermore, it is well known that ionic copper is able to catalyse peroxidation in model lipid systems (Wills, 1965). Taking these data into account, it is suggested that Cu(II) ions may be able to initiate lipid peroxidation in RBC by direct interaction with the membrane components.

On the other hand, the interaction between Cu(II) and HbO₂ could also be an important factor in the haemolytic action of copper. Haemoglobin autooxidation strongly increases in the presence of Cu(II) ions (Rifkind, 1974; Winterbourn & Car-

rell, 1977), and both products of Hb oxidation, methaemoglobin and O₂^{•-}, are able to induce lipid peroxidation (Tappel, 1955; Tarladgis, 1961; Miura & Ogiso, 1981). It seems likely that MetHb may cause peroxidation in RBC after coming into contact with the membrane lipids. Normally only a small amount of Hb in RBC is membrane-bound, and the level of lipid peroxidation is very low. Therefore we suggest that the entry of ionic copper into the cytosol may result in an increased binding of Hb to the membrane, which creates more favourable conditions for the initiation of peroxidation.

The aim of the present study was to investigate the binding of Hb to the RBC membrane in the presence of CuCl₂ in the hope that the results obtained will help our understanding of the mechanism of copper-induced peroxidation in RBC.

Materials and methods

RBC membrane preparations

Guinea-pig RBC were washed with 0.15M-NaCl/5mM-phosphate buffer, pH 8.0, and were haemolysed with 5mM-phosphate buffer in a ratio of 1:40 (v/v). The membranes were washed four times with the same buffer at 4°C. Thus-prepared

Abbreviations used: RBC, red blood cell(s); Hb, haemoglobin; MetHb, methaemoglobin; AS, 12-(9-anthroyl)stearic acid; O₂^{•-}, superoxide radical.

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ghosts contained less than 0.5% of the haemoglobin to be added during the binding experiments. RBC ghosts in 0.15M-NaCl were counted with a cell counter (Laborscale, type PSL-I).

Inside-out and right-side-out resealed ghosts were prepared by the method of Steck (1974), and the assay of sidedness and sealing was performed by using the marker enzymes acetylcholinesterase (EC 3.1.1.7) and glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) respectively (Steck, 1974; Steck & Kant, 1974).

Other preparations and reagents

HbO₂ and MetHb were prepared as described previously (Ribarov *et al.*, 1981). Results presented were obtained with guinea-pig RBC, but similar results were found when human RBC were used. All reagents were of analytical grade and water was twice-glass-distilled.

Fluorescence method for measurement of Hb binding to RBC membranes

For measurement of Hb binding to RBC membrane the method of Shaklai *et al.* (1977a,b) was used. AS (1:200, w/w, with respect to the estimated lipids) was added to the membrane suspension and incubated for 1 h. The membranes were then washed with 5mM-phosphate buffer, pH 8.0, to eliminate the unbound AS in the water phase.

The binding of both HbO₂ and MetHb to the RBC membrane at low and at high ionic strength of the medium was carried out with membranes suspended in 5mM-phosphate buffer and 0.15M-NaCl in 5mM-phosphate buffer respectively (pH as indicated in Table 1 below).

The quenching effect of HbO₂ and MetHb on the fluorescence differed negligibly. The results presented are from experiments with HbO₂.

The fluorescence emission was measured at 450nm and the excitation wavelength was 360nm (the bandwidth for both wavelengths was 12nm).

Results and discussion

As Table 1 shows, the addition of Hb to the AS-labelled ghosts, suspended in 5mM-phosphate buffer (low ionic strength of the medium), led to some quenching of their fluorescence. The subsequent elevation of the ionic strength by addition of NaCl caused a marked restoration of fluorescence. Both effects depended strongly on pH, thus indicating that, in the absence of CuCl₂, the Hb binding to the RBC membrane is electrostatic. In the presence of CuCl₂ the quenching of the fluorescence by Hb increased significantly in a relatively pH-independent manner. This suggested that Cu(II) ions may cause an additional binding of Hb to the RBC that is non-electrostatic in nature.

The addition of CuCl₂ to haemoglobin-free membrane suspension resulted in a decrease of the fluorescence intensity of approx. 20%. The effect was independent of pH and the ionic strength of the medium.

When Hb was added to RBC ghosts suspended in 0.15M-NaCl and buffered with 5mM-phosphate buffer, very little quenching of fluorescence was observed. This indicated that, at high ionic strength and in the absence of CuCl₂ in the suspending medium, the amount of the membrane-bound Hb is negligible. In this case significantly more Hb molecules become bound after addition of CuCl₂ and the effect remained noticeable even at high pH values.

Two classes of sites able to bind Hb in RBC membrane were demonstrated: high-affinity sites with a binding constant of $1 \times 10^8 \text{ M}^{-1}$, characteristic of the inner surface of the membrane, and low-affinity sites with a binding constant of $6 \times 10^6 \text{ M}^{-1}$, which are non-specific (Shaklai *et al.*, 1977b). On the basis of these results we suggest that, in the presence of CuCl₂, the two surfaces of the RBC membrane may have differing capacities for binding of Hb. This possibility was tested and discarded after investigation of the Hb quenching effect on the fluorescence of two different membrane preparations marked with AS: inside-out and right-side-out resealed ghosts. As Table 1 shows, approximately equal quenching of the fluorescence of both membrane preparations by Hb was observed in the presence of CuCl₂. This results suggested that, in the presence of ionic copper, the haemoglobin binding to the RBC membrane is rather non-specific.

The Hb quenching effect on the membrane fluorescence depended on the CuCl₂ concentration. When the membrane suspension with low ionic strength (5mM-phosphate buffer, pH 7.4) contained 0.2 μM -Hb, the amount of the membrane-bound Hb increased with the increase of the CuCl₂ concentration up to 10 μM .

At high ionic strength and at pH 7.4 the addition of increasing quantities of Hb to the RBC ghosts resulted in a progressive augmentation of the amount of the membrane-bound Hb, until saturation was achieved (results not shown). In the presence of CuCl₂, the saturation level was significantly higher, and it was reached at a lower concentration of added Hb.

Taken together, the data obtained show that, in the presence of CuCl₂, the Hb quenching of the RBC membrane fluorescence significantly increased. This effect of CuCl₂ is most likely due to an additional binding of Hb to the RBC membrane, which is almost independent of pH and ionic strength of the medium. The increased binding of Hb to the membrane seems to be very

Table 1. *Hb quenching of the fluorescence of AS-labelled RBC membrane preparations*

The concentration of the non-resealed-ghost preparation was 5×10^6 ghosts/ml. The right-side-out and inside-out resealed ghost preparations were adjusted to a concentration at which their fluorescence was equal to the fluorescence of the non-resealed-ghost preparation. The suspending medium was 5 mM-phosphate buffer (L) or 0.15M-NaCl in 5 mM-phosphate buffer (H). The quenching was performed by addition of 0.2 μ M-Hb or 0.2 μ M-Hb plus 50 μ M-CuCl₂. To increase the ionic strength of the medium, NaCl was added to a concentration of 82 mM. The initial fluorescence of all membrane preparations was 100 arbitrary units. Three experiments with three replicates were carried out. Results are means $\pm$ S.E.M.

Suspending medium		Fluorescence (arbitrary units)											
		Non-sealed ghosts						Resealed ghosts					
		Quenching by addition of:			Restoration after addition of NaCl:			Right-side out; quenching by addition of:			Inside-out; quenching by addition of:		
pH	Ionic strength	Hb	Hb + CuCl ₂	Hb	Hb + CuCl ₂	Hb	Hb + CuCl ₂	Hb	Hb + CuCl ₂	Hb	Hb	Hb + CuCl ₂	
6.0	L	44.7 $\pm$ 0.6	25.3 $\pm$ 0.5	84.5 $\pm$ 1.0	30.1 $\pm$ 1.0	60.7 $\pm$ 2.8	36.7 $\pm$ 2.8	67.0 $\pm$ 4.0	46.0 $\pm$ 4.2				
	H	97.5 $\pm$ 0.5	45.0 $\pm$ 1.0	—	—	—	—	—	—	—	—	—	
6.5	L	58.0 $\pm$ 0.6	34.0 $\pm$ 0.4	85.3 $\pm$ 1.0	36.6 $\pm$ 0.8	67.4 $\pm$ 1.2	37.4 $\pm$ 1.4	67.4 $\pm$ 3.0	47.4 $\pm$ 2.9				
	H	97.5 $\pm$ 0.5	52.5 $\pm$ 0.5	—	—	—	—	—	—	—	—	—	
7.0	L	81.4 $\pm$ 0.8	35.4 $\pm$ 0.5	86.3 $\pm$ 1.0	36.6 $\pm$ 0.8	83.4 $\pm$ 3.0	40.0 $\pm$ 1.9	76.0 $\pm$ 3.8	48.4 $\pm$ 4.5				
	H	97.9 $\pm$ 0.4	53.5 $\pm$ 0.6	—	—	—	—	—	—	—	—	—	
7.4	L	88.7 $\pm$ 1.0	40.0 $\pm$ 0.7	89.6 $\pm$ 1.0	42.0 $\pm$ 0.6	88.7 $\pm$ 2.8	40.7 $\pm$ 2.6	85.0 $\pm$ 2.7	50.0 $\pm$ 4.5				
	H	97.5 $\pm$ 0.5	55.0 $\pm$ 0.4	—	—	—	—	—	—	—	—	—	
8.0	L	89.4 $\pm$ 1.0	46.7 $\pm$ 0.6	89.6 $\pm$ 0.5	48.3 $\pm$ 0.3	90.7 $\pm$ 2.4	48.0 $\pm$ 2.2	88.4 $\pm$ 4.0	52.7 $\pm$ 3.2				
	H	98.0 $\pm$ 0.1	62.1 $\pm$ 0.7	—	—	—	—	—	—	—	—	—	

important, as it possibly creates more favourable conditions for initiation and development of lipid peroxidation in RBC membrane.

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