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BINDING OF IRON TO HUMAN RED BLOOD CELL MEMBRANES

EROL BAYSAL, STEPHEN GENE SULLIVAN[1] and ARNOLD STERN

Department of Pharmacology, New York University School of Medicine, 550 First Avenue, New York, NY 10016, USA, and [1]Department of Psychiatry, Beth Israel Medical Center, First Avenue at 16th Street, New York, NY 10003, USA

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The binding of Fe^{3+} to red cell membranes was studied in a system in which lipid peroxidation was proportional to Fe^{3+} concentration. Binding of Fe^{3+} was evaluated by labeling with $^{59}FeCl_3$ and measurement of NMR water-proton relaxation times. Labeling with ^{59}Fe showed that 95% of the Fe^{3+} was membrane bound at 100 μM $FeCl_3$ in a 1.5 mg protein/ml membrane suspension. Both spin-lattice (T_1) and spin-spin (T_2) relaxation times decreased with increasing Fe^{3+} concentration. Addition of red cell membrane suspensions largely prevents the Fe^{3+} effect on relaxation times. Charge transfer to Fe^{3+} may occur at the membrane binding site with resultant decrease in the Fe^{3+} effect on water-proton relaxation times. These studies support the hypothesis that Fe^{3+} binds to the membrane and generates free radicals at the binding site.

KEY WORDS: Iron binding, red cell membranes, NMR, Spin-lattice relaxation time, Spin-spin relaxation time

ABBREVIATIONS: TCA, trichloroacetic acid; BHT, butylated hydroxytoluene; TBAR, thiobarbituric acid reactive products; ms, milliseconds

INTRODUCTION

The role of iron in the peroxidation reactions within biological membranes has been the subject of intense research in a variety of systems. Extensive work has been reported on iron-mediated lipid peroxidration in liposomes,[1,2] microsomes[3] and synaptosomes.[4] Red blood cells and their membranes have also been used as model systems to elucidate the iron-induced membrane damage.[5] No attempt was made to locate the sites of iron binding in these studies. Iron may be sequestered in the membrane in the form of hemichrome,[6,7] diffuse into the bulk lipid phase, exist in the surrounding aqueous medium, or precipitate out of solution as insoluble material. Study of the binding of iron in biological and non-biological systems is important to understanding the mechanism of iron-dependent lipid peroxidation.

Fe^{3+} is known to catalyze the decomposition of lipid hydroperoxides[8]. Metal-stimulated lipid peroxidation may be dominated by secondary reactions rather than reactions of initiation.[9,10] Studies of iron binding to biological membranes may be helpful in elucidating mechanisms of iron-stimulated lipid peroxidation. In this work we have evaluated iron-binding in an iron-red cell membrane system in which lipid

Correspondence to: Arnold Stern, MD, PhD Department of Pharmacology New York University Medical Center 550 First Avenue New York, NY 10016, USA Tel: (212) 340 7118 Fax: (212) 340 7133

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peroxidation is proportional to Fe^{3+} concentration. Iron binding to red cell membranes was evaluated by labeling with $^{59}FeCl_3$ and the measurement of NMR water-proton relaxation times.

MATERIALS AND METHODS

Preparation and incubation of RBC membranes

Normal adult human blood samples were collected daily into a test tube containing a small amount of 3.8% buffered sodium citrate. After centrifugation at room temperature for 5 min at 2500 rpm, the plasma and buffy coat were removed and red cells were washed three times with 0.9% NaCl.

Erythrocyte membranes were prepared by hypo-osmotic hemolysis at pH 7.5 on the day of the experiment according to Dodge *et al.*[11] and resuspended in 5 mM Tris-HCl, pH 7.4. No pH change was observed before and after incubation. Small aliquots of iron were added to the test samples so that the final membrane protein concentration did not change. The reaction was carried out at 37°C by incubation in a shaking water bath. Protein concentrations were estimated by the method of Lowry *et al.*[12] using bovine serum albumin as a standard.

Preparation of stock iron solutions

Stock solutions were prepared by dissolving $FeCl_3$ in deionized water immediately prior to use. No precipitation of polynuclear complexes due to phosphate contaminants was observed in the stock solutions or incubation mixtures. This was confirmed by addition of ^{59}Fe to these solutions. No decrease of ^{59}Fe occurred after centrifugation of these solutions.

Measurements of lipid peroxidation

Aliquots were removed for the TBAR-test according to the modified method of Kumar *et al.*[13] Peroxidation of unsaturated membrane lipids was measured by the TBAR assay in which malondialdehyde and other by-products of peroxide decomposition are reacted with 2-thiobarbituric acid to produce colored adducts. The assay was carried out in the presence of BHT to prevent autoxidation of remaining lipids. Membranes were precipitated with TCA and boiled in the presence of TBA and BHT for 20 minutes. The pink chromophore was measured at 532 nm using a Cary-14 spectrophotometer.

Labeling of membranes with $^{59}FeCl_3$

Membranes (1.5 mg protein/ml) were labeled by incubating for 30 min at 37°C with 10 and 100 μM $FeCl_3$ containing ^{59}Fe (approximately 50000 cpm per 0.5 ml (28.05 mCi/g, New England Nuclear)). After the incubation, membranes were precipitated with ice-cold TCA (final concentration 17%) and centrifuged at 16500 rpm for 10 min. The supernatant was analyzed for radioactivity using a Gamma 4000 counter (Beckman Scientific Instruments). The pellet was washed once with buffer in order to remove residual radioactivity. The bottom ends of each tube containing the

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precipitated pellet was cut off and analyzed for radioactivity. Results were not corrected for unspecific binding of radioactivity to the plastic since the amount of radioactivity bound to the walls of the tubes was negligible.

Measurement of NMR water-proton relaxation times

Measurements of NMR water-proton relaxation times were carried out using a Seimco MicroPulse NMR Spectrometer (New Kensington, PA) operating at 13.25 MHz. Measurements were made at a sample and probe temperature of 29°C on 0.5 ml sample volumes. Spin-lattice (T_1) relaxation times were measured using a steady state sequence[14] described by Freeman and Hill.[15] Spin-spin (T_2) relaxation times were measured using the modification by Meiboom and Gill[16] of the spin-echo sequence of Carr and Purcell.[17]

RESULTS

Table 1 shows the degree of lipid peroxidation in human red blood cell membranes as measured by formation of TBAR. Lipid peroxidation was found to be proportional to the Fe^{3+} concentration. Under the same conditions, iron binding to red cell membranes was measured by labeling with $^{59}FeCl_3$. After incubation, membrane suspensions were centrifuged to separate the bound from free iron and the distribution of radioactive iron was measured. Counts were recorded in the pellet and in the supernatant (bound and free iron respectively) and the results were tabulated in terms of percent of iron bound to the membrane and nmoles of iron bound per mg protein. The data shows that nearly all of the iron was bound to the membrane at both 10 μM and 100 μM $FeCl_3$.

In addition to radioactive labeling of membranes, binding of iron to red cell membranes was also observed by the measurement of NMR water-proton relaxation

TABLE I

The relationship of iron concentration to TBAR formation and to iron binding in red blood cell membrane suspensions.

$FeCl_3$	TBAR	IRON BINDING	
(μM)	(A_{532nm})	Percent of iron bound to the membrane	nmoles of iron bound per mg protein
0	0.010	0	0
10	0.057	94.3	6.3
100	0.170	95.3	63.5

Suspensions of membranes (1.5 mg protein/ml) in 5 mM Tris-HCl, pH 7.4, were incubated with various concentrations of $FeCl_3$ for 60 min at 37°C in a shaking water bath followed by determination of TBAR as described in Materials and Methods. Suspensions of membranes in 5 mM Tris-HCl, pH 7.4, with $FeCl_3$ labeled with ^{59}Fe were incubated for 30 min at 37°C in a shaking water bath followed by measurement of binding of radiolabeled iron as described in Materials and Methods. Tubes containing $^{59}FeCl_3$ in the *absence* of membranes showed that 99.3% radioactivity remains in the buffer and 0.7% on the walls of tubes. The small percentage of radioactivity found in the supernatant of test samples (approx. 6%) may be partially attributed to ^{59}Fe bound to the tiny membrane fragments too small to be precipitated during centrifugation. The results were reproducible in three experiments and samples with the same treatment varied not more than 4% from one experiment to the other.

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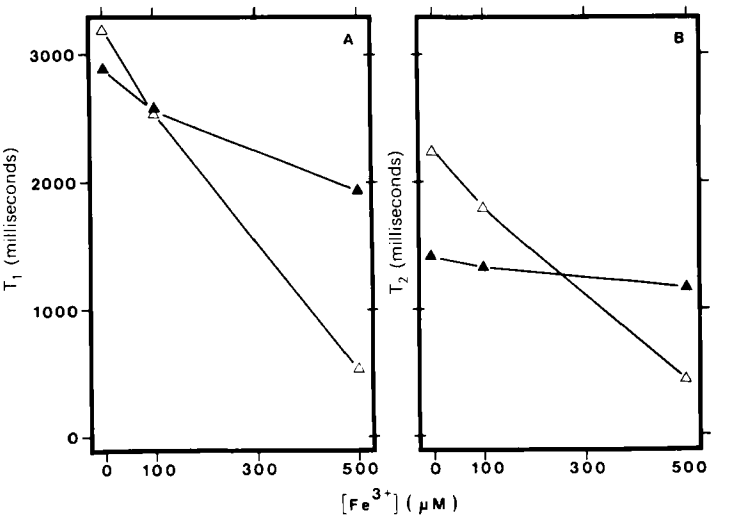

FIGURE 1 NMR water-proton relaxation times of $FeCl_3$ solutions in the presence and absence of red cell membrane suspensions. Fig. 1A, spin-lattice (T_1) relaxation times; Fig. 1B, spin-spin (T_2) relaxation times. (△) $FeCl_3$ in water; (▲) $FeCl_3$ in 1.5 mg protein/ml membrane suspensions in 5 mM Tris-HCl, pH 7.4.

times. Fig. 1A shows the water-proton spin-lattice relaxation time (T_1) of water and red cell membrane suspensions at 0, 100 μM and 500 μM $FeCl_3$. Water had a T_1 time of 3200 ms. Red cell membranes lowered the T_1 time to 2900 ms. The addition of Fe^{3+} to water in increasing concentrations resulted in significant decrease in the T_1 time to 500 ms with 500 μM Fe^{3+}. The addition of Fe^{3+} to membrane suspensions also caused a decrease in T_1 time in a concentration-dependent manner. The effects of Fe^{3+} in decreasing the T_1 time however was less pronounced in the presence of red cell membranes. Fig. 1B presents the spin-spin relaxation time (T_2) of water and red cell membrane suspensions at identical iron concentrations. Water had a T_2 time of 2200 ms. Red cell membranes lowered the T_2 time to 1400 ms. Red cell membranes had a much greater effect on T_2 time than on T_1 time. As observed with T_1 times, Fe^{3+} decreased the T_2 time of water while the presence of red cell membranes largely prevented the Fe^{3+} effect.

DISCUSSION

Binding of iron to red cell membranes was studied in a system in which the degree of lipid peroxidation was proportional to $FeCl_3$ concentration. Approximately 95% of the metal ion remains bound to the membranes during centrifugation as measured by labeling of the membranes with ^{59}Fe. These studies support the hypothesis that iron is bound to the membrane and that the subsequent generation of free radicals occurs at the binding site. It seems conceivable that iron is bound in a similar manner in other commonly studied biological and non-biological systems.[1-4]

Increases in concentration of macromolecules and paramagnetic metal content can both decrease water-proton relaxation times.[18] Red cell membranes decreased both T_1

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and T_2 relaxation times reflecting mainly macromolecular effects. Decreases of T_1 and T_2 relaxation times by addition of Fe^{3+} are due to paramagnetic relaxation. Blocking of the Fe^{3+}-induced paramagnetic relaxation by red cell membranes implies decrease in the magnetic susceptibility of the solution by binding of Fe^{3+} to the α membrane. One possible interpretation is that the magnetic susceptibility is decreased by transfer of charge to Fe^{3+} from electrophilic centers on the membrane. This is consistent with the recent findings that α-tocopherol incorporated into artificial liposomal membranes reduced Fe^{3+} rapidly to a more reactive Fe^{2+} ion.[19] Since a-topcopherol is known to exist in red cell membranes in large quantities it is possible that a transfer of charge from α-tocopherol to Fe^{3+} occurs in this system. Although no characterization of the iron binding sites has been made in this study, we believe that carboxyl groups of sialic acids, sulfate groups[20] of glycolipids and glycoproteins and sulfin and sulfon groups[21] together with the phosphate head groups of the phospholipids may constitute the major binding sites on red blood cell membrane surface.

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