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Inhibition of the erythrocyte (Ca^{2+} + Mg^{2+})-ATPase by nonheme iron

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The erythrocyte calmodulin-stimulated (Ca^{2+} + Mg^{2+})-ATPase (CaM-ATPase), an integral membrane protein, is inhibited in different types of congenital hemolytic anemias for which oxidative processes appear as a common feature. The oxidation of hemoglobin and its degradation lead to the accumulation of ferric heme (hemin) and nonheme iron in the red cell. We have shown previously that hemin inhibits the activity of the enzyme of normal erythrocyte (Leclerc et al. (1988) Biochim. Biophys. Acta, 946, 49–56) involving an oxidation of thiol groups. The present study demonstrates that nonheme iron also inhibits the CaM-ATPase activity. In contrast with hemin, the inhibition of the enzyme induced by the nonheme treatment is prevented by butylated hydroxytoluene, a protecting agent of unsaturated phospholipid peroxidations, while dithiothreitol, a reducing agent of protein disulfide bridges, does not restore the activity of the enzyme. We conclude that nonheme iron inhibits the enzyme at least in part, through the peroxidation of phospholipids of the membrane bilayer.

Introduction

The erythrocyte CaM-ATPase is inhibited in different types of hemolytic anemia such as sickle-cell disease or glucose 6-phosphate dehydrogenase deficiency, in which oxidative processes have been well documented [1–3]. The oxidation of hemoglobin to methemoglobin and its degradation leads to the accumulation of hemin and free iron in the red cell membrane [4,5]. We have shown recently that the CaM-ATPase is inhibited by hemin, the ferric protoporphyrin IX complex [6]. Because the inhibition of the enzyme was partially reversed by dithiothreitol, a reducing agent of disulfide bonds, it was concluded that hemin induces the oxidation of thiol groups in the protein. A similar conclusion was reported for membrane skeleton proteins [7]. Nonheme iron is likely present in the cell as an Fe(III)/Fe(II) mixture, a Fenton reagent and a potent generating system of reactive oxygen radicals [8,9]. To our knowledge, no study has yet reported the effect of nonheme iron on the activity of the integral CaM-ATPase and other cation pumps of the erythrocyte membrane. Because of its potential interest in the regulation of intracellular Ca^{2+} in sickle-cell disease as well as in nonheme iron overload pathologies, we have investigated the effect of iron on the activity of the CaM-ATPase of normal adult red cell membranes.

Material and Methods

Chemicals

Vanadium-free ATP, hog brain calmodulin (CaM) and dithiothreitol (DTT) were purchased from Boehringer (Mannheim, F.R.G.); butylated hydroxytoluene (BHT) and 4,7-diphenyl-1,10-phenanthroline from Sigma (St. Louis, U.S.A.), bovine serum albumin (BSA) from Serva (Heidelberg, F.R.G.). Ammonium ferric iron citrate was a product from Merck (Darmstadt, F.R.G.). Deferoxamine was from Ciba-Geigy (Basel, Switzerland). All chemicals were of the highest purity grade available.

Preparation of the erythrocyte membranes

Red cell membranes were prepared from bank blood according to the method described by Farrance and Vincenzi [10] and kept frozen at −80 °C in a histidine-imidazole buffer 40 mM (pH 7.4). Before use the membranes were washed thrice with saline solution to

Abbreviations: CaM-ATPase, calmodulin-stimulated (Ca^{2+} + Mg^{2+})-ATPase; BHT, butylated hydroxytoluene; BSA, bovine serum albumin; DTT, dithiothreitol.

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eliminate the buffering system. Previous experiments did not show evidence for a difference of the activity of the enzyme between the stored and freshly prepared membranes. Following the recommendations of Braughler et al. [8] no buffering system was used in the presence of nonheme iron to avoid a possible electron trapping effect. For this reason, the membranes were treated with ammonium iron citrate, a ferric iron complex, soluble in water at pH 7.

Estimation of Fe(III) and Fe(II) concentration

Ferric and ferrous iron concentrations were estimated from the absorbance of the colored complexes which are formed in the presence of deferoxamine (Fe(III)) and diphenyl-phenanthroline (Fe(II)). The absorbances were read at 430 nm for the ferric and 510 nm for the ferrous complexes, respectively [11]. A calibration curve was first established with ferric or ferrous iron salt solutions.

Iron treatment

Membrane suspensions (1 mg protein/ml) containing 1 mg saponin per mg of protein, were incubated in the presence of varying concentrations of ammonium iron citrate for different periods of time, in saline solution at 37 °C and pH 7.4.

Measurements

The activity of the CaM-ATPase was measured as described previously [1]. Inorganic phosphate produced by the hydrolysis of ATP was measured according to Kallner [12]. Control experiments were run to check for the possible influence of citrate on the enzyme activity due to Ca^{2+} chelate formation. No difference between the controls and the membranes treated with 200 μM iron-free citrate membranes was observed. The CaM-ATPase activities were 97 ± 4 and 94 ± 6 nmol P_i/min per mg protein (means of eight measurements), respectively. The concentrations of ATP, Ca^{2+} and CaM in the assay mixture were varied in order to obtain maximal activities of the enzyme. The iron treatment did not modify our optimum routine conditions [1]. Membrane protein determination was carried out according to Bradford [13] using bovine serum albumin (BSA) as a standard. To understand more precisely the mechanism(s) by which iron treatment alters the CaM-ATPase activity, experiments were run using different reagents: (i) deferoxamine, an Fe(III) chelator was added at the onset of the iron treatment; (ii) BHT, a protecting agent of membrane phospholipid peroxidations, was added at the onset of the experiment. Preliminary studies had shown that BHT does not change the concentration ratio between Fe(III) and Fe(II) in the incubation medium in the absence of membranes; (iii) DTT was added at the end of the iron treatment period to check for a possible oxidation of thiol groups – this was followed by a 60 min incubation period before the enzyme activity assay.

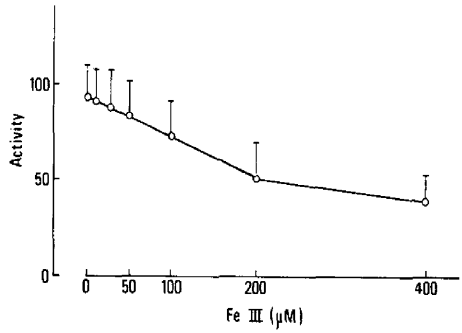

Fig. 1. Concentration-dependent inhibition of human erythrocyte CaM-ATPase activity by nonheme iron. Red blood cell membranes (1 mg protein/ml) were incubated at 37 °C for 60 min in the presence of varying concentrations of nonheme iron. CaM-ATPase activity is expressed as nmol P_i/min per mg of membrane proteins. Results represent the average of three independent determinations. The bars are ± 1 S.E.

Results

Red cell membranes treated with the ferric iron complex exhibit an inhibition of the CaM-ATPase activity. As shown in Figs. 1 and 2, the inhibition depends on the concentration of iron and the duration of the treatment. For 200 μM Fe(III) concentration a 50% inhibition was reached after a 60 min incubation period. Beyond this concentration, the slope of the enzyme activity versus Fe(III) concentration curve is diminished. These results show that the CaM-ATPase activity decreased rapidly, indicative of a potent inhibitory effect of the metal.

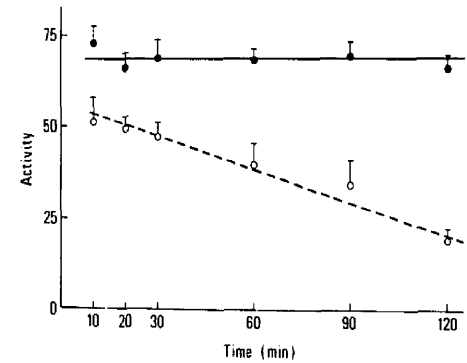

Fig. 2. Time-dependent inhibition of the erythrocyte CaM-ATPase by nonheme iron. Red blood cell membranes (1 mg protein/ml) were incubated at 37 °C in the presence of 200 μM nonheme iron. CaM-ATPase activity is expressed as nmol P_i/min per mg of membrane protein. The values are the means of three experiments (± 1 S.E.).

TABLE I

Concentrations (μM) of ferric and ferrous iron in the incubation medium

Concentrations were determined in the medium before and after 60 and 120 min of incubation with the red cell membranes (1 mg protein/ml). Results are the means of five measurements. Typical errors were ±10% for Fe(III) and ±20% for Fe(II) measurements.

Iron	Incubation time (min)		
	0	60	120
Fe(III)	200	193	188
Fe(II)	26	77	75
% Fe(III)	88	70	71

The initiating step for the production of oxygen radical species by iron (or copper) necessitates the existence of a redox cycle of the transition metal, known as the Fenton reaction [9]. In the present study, ferric iron was the original source of iron in the medium. However, the measurements of ferric and ferrous iron concentrations demonstrated the presence of approx. 10% ferrous iron in the freshly prepared iron solution (Table I). In the presence of red cell membranes, an increase of ferrous iron concentration was observed with the disappearance of the ferric iron. This ferric/ferrous iron oxido-reduction couple is likely responsible for the production of oxygen activated species and for the inhibition of the enzyme through oxidative processes. This is supported by the observation that the addition of deferoxamine, a specific ferric iron chelating agent at the onset of the membrane iron treatment completely prevented the inhibition of the CaM-ATPase activity (Table II). Fig. 3 shows that the inhibitory effect of the iron treatment (50 μM) is nearly completely prevented by 1 mM BHT. This result indicates that the oxidative inhibition of the CaM-ATPase is related more specifically to lipid peroxidations. At higher concentrations of iron, BHT provided only a partial protection (30%) of the enzyme activity. Increasing the concentration of BHT up to 10 mM did not improve this result, suggesting that secondary mechanisms, possibly different from lipid peroxidations, contribute to the inhibition of the enzyme. The formation of disulfide bridges was ruled out by the lack of reactivation of the CaM-ATPase activity after the addition of DTT. Deferoxamine was also added at the end of the iron treatment period in an attempt to displace ferric nonheme iron from possible binding sites within the protein. No restoration of the activity of the enzyme was observed (results not shown).

TABLE II

The effect of deferoxamine on the inhibition of CaM-ATPase

Deferoxamine 4 mM (final concentration) was added at the onset of the incubation period in the presence of 400 μM nonheme iron. Results represent the average of three experiments. Typical errors were ±20%.

	CaM-ATPase activity	
	nmol P_i/min per mg protein	% activity/ control
Control	76	100
+ Deferoxamine	75	99
+ Iron	15	20
+ Iron + Deferoxamine	76	100

TABLE III

The protective effect of BHT on the inhibition of red cell membrane CaM-ATPase activity at high nonheme iron concentrations

BHT (1 mM) was added at the onset of the 60 min incubation period of the red cell membrane (1 mg protein/ml) in the absence or presence of increasing concentration of nonheme iron. Activities in the control experiments (without iron) were 134 ± 13 (− BHT) and 125 ± 14 (+ BHT) nM P_i/mg protein per min. Results are the average of four experiments. Typical errors were ± 10%.

Iron μM	%CaM-ATPase activity	
	− BHT	+ BHT
0	100	100
100	55	81
200	52	82
400	36	65

Discussion

We have previously shown that hemin inhibits the erythrocyte CaM-ATPase [6] through an interaction leading to the oxidation of thiol groups in the protein

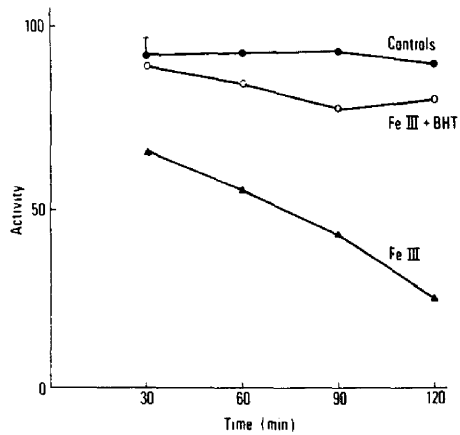

Fig. 3. Protection of the CaM-ATPase activity by BHT. Red blood cell membranes (1 mg protein/ml) were incubated in the presence of 50 μM nonheme iron and 1 mM BHT at 37°C for different periods of time. Control experiments were run without and with 50 μM iron. The results are the average of three experiments. Typical errors were 10%.

[14]. It was also shown that neither protoporphyrin IX nor liganded ferric or ferrous heme alter the activity of the enzyme, pointing to the predominant role of unliganded ferric iron as a strong catalyst for the inhibitory effect. The present results demonstrate that nonheme iron inhibits the CaM-ATPase in a time- and concentration-dependent process, as observed for hemin. However, the pathway for the inhibition by nonheme iron appears different from that postulated for hemin. BHT protects the enzyme from the inhibition by nonheme iron, suggesting the role of lipid peroxidations as the primary targets of the activated oxidative species. By contrast, BHT had no effect on the hemin CaM-ATPase inhibition [6].

The oxidation of membrane components by transition metals requires the presence of a redox cycle which initiates the Fenton reaction [15]. Our experimental conditions satisfy this requisite. Fe(III) and Fe(II) were present in our incubation medium. Chelating ferric iron ions by deferoxamine or reduction to ferrous iron at the onset of the reaction procedure protected the enzyme from the inhibitory process. This demonstrates that ferrous iron per se does not inhibit the enzyme activity.

Kuross et al. [16] have pointed out that the hydroperoxyl radical produced through the Fenton reaction is such a highly reactive species that it should interact with the membrane components located in the immediate vicinity of its production. Phosphatidylserine, a phospholipid exclusively located in the inner leaflet of the membrane, is a good candidate for the lipid iron-mediated peroxidation [16]. This is of interest, since the red cell CaM-ATPase protrudes into the cytosol and is very sensitive to its phospholipid environment. We postulate that the enzyme inhibition is likely due to the formation of irreversible cross-links induced by malondialdehyde, the main end product of lipid peroxidation. Previous studies support this hypothesis, since nonheme iron increases the production of malondialdehyde in red cell membrane suspensions [16] and leads to an irreversible inhibition of the enzyme activity [1]. Due to the inhomogeneous repartition of heme and nonheme iron in the vicinity of the membrane, it is plausible that local concentrations of iron as high as those used in this study may be attained in vivo in various hemolytic syndromes or iron overload pathologies.

Acknowledgments

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