

Desferrioxamine as a lipid chain-breaking antioxidant in sickle erythrocyte membranes

Andrew Hartley¹, Michael Davies² and Catherine Rice-Evans¹

¹*Department of Biochemistry and Chemistry, Royal Free Hospital School of Medicine, University of London, Rowland Hill Street, London NW3 2PF* and ²*Department of Chemistry, University of York, Heslington, York YO1 5DD, UK*

Received 2 March 1990; revised version received 21 March 1990

The mechanism of action of desferrioxamine in the inhibition of the catalysis of iron-induced oxidative damage has been ascribed to its ability to chelate available ferric ion ($K_b = 10^{31}$). However, recent work has proposed that the trihydroxamate moiety of desferrioxamine can also be involved in electron transfer reactions involving the superoxide radical, peroxidase/hydrogen peroxide mixtures and ferryl myoglobin radicals. In this study we report evidence for the ability of desferrioxamine to inhibit peroxidative damage to pathological membranes with which non-haem iron is associated through a mechanism of action as a lipid chain breaking antioxidant, independently of its iron chelating properties.

Desferrioxamine; Iron; Antioxidant; Sickle erythrocyte membrane; Electron spin resonance spectroscopy

1. INTRODUCTION

Desferrioxamine is currently the only iron chelator in widespread clinical use to treat states of iron overload. It chelates available ferric iron [1] rendering it unable to participate in redox cycling and thereby catalyse potentially damaging redox reactions. Iron within haem is not chelatable.

Abnormal iron-containing species have been described both within the intact sickle erythrocyte and associated with the isolated membrane [2–9], the majority of the latter being mainly non-haem in nature [8,9]. Studies *in vitro* have suggested that such iron species may contribute towards the amplification and propagation of peroxidative damage to lipids in sickle cell membranes by catalysing the breakdown of lipid hydroperoxides, these responses being suppressed on incorporation of antioxidants such as tocopherol [4]. Desferrioxamine has also been reported to suppress the incubation-induced exacerbation of lipid peroxidation in sickle cell membranes [4]. This observation was interpreted as an indication of the availability of chelatable iron in the sickle cell membrane and its involvement in the mechanism of the peroxidative degradation of the membrane lipids.

Recent work has proposed that the trihydroxamate moiety of desferrioxamine, the centre involved in the binding of the iron, can be involved in electron transfer through its ability to donate atoms or electrons to

horseradish peroxidase/hydrogen peroxide mixtures [10,11] and to ferryl myoglobin [11–13] and to interact with superoxide radicals [14]. The oxidation of oxyhaemoglobin [15,16] and oxyleghaemoglobin [17] by desferrioxamine has also been documented.

Here we report findings consistent with the ability of desferrioxamine to act as a chain-breaking antioxidant of peroxidising lipids in biological membranes independently of its iron chelating properties. The results provide evidence for the formation of the desferrioxamine nitroxide radical suggesting that the iron-mediated propagation of lipid peroxidation may be inhibited in sickle erythrocyte membranes by a mechanism of hydrogen donation from the trihydroxamate centre.

2. MATERIALS AND METHODS

All reagents were obtained from Sigma unless otherwise stated. Desferrioxamine was used as the mesylate derivative (Desferal, Ciba-Geigy). Horse spleen ferritin in isotonic saline was dialysed extensively against conalbumin (50 mg/ml) before use [18].

Erythrocyte membranes were prepared from sickle and normal erythrocytes by the method of Dodge *et al.* [19] at pH 7.4 and not subsequently resealed. Membranes were quantified on the basis of protein content using the method of Lowry *et al.* [20] with bovine serum albumin as a standard.

Membrane-associated non-haem iron was assayed utilising a modification of the ferrozine assay [21] at pH 7.4 in 10 mM phosphate-buffered saline with sodium dodecyl sulphate (0.67% w/v) to solubilise membranes. The absorbance at 562 nm was monitored as a function of time. The reaction between ferrozine and membrane-associated non-haem iron was complete after 48 h. Concentrations of non-haem iron were calculated by use of standard curves prepared using ferrous sulphate or ferric chloride in 10 mM hydrochloric acid. Equivalent results were obtained making use of the molar extinction

Correspondence address: C. Rice-Evans, Department of Chemistry and Biochemistry, Royal Free Hospital School of Medicine, University of London, Rowland Hill Street, London NW3 2PF, UK

coefficient for the ferrozine:iron complex of 2.79×10^4 at 562 nm [22]. The response of ferritin in this assay was also examined.

The ability of membrane-associated iron components to catalyse the breakdown of organic hydroperoxides to alkoxyl and peroxy radical species was assessed using electron spin resonance spectroscopy applying the spin trap 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO). Sick and normal erythrocyte membranes (1.7 mg protein/ml) were incubated at 22°C with *t*-butyl hydroperoxide (10 mM) and DMPO (40 mM) in an aqueous sample cell within the cavity of a Bruker ESP 300 electron spin resonance spectrometer equipped with 100 kHz modulation and a Bruker ER035M gaussmeter for field calibration. Hyperfine coupling constants were measured directly from the field scan and compared with previously reported values [23]. Spectra were recorded 90 s after mixing and, where necessary, scanned repeatedly with 10 s intervals. Spin adduct concentrations were obtained by measuring the peak to peak line heights of signals recorded at identical times after initiation of the reaction using identical machine settings.

Ferritin, at concentrations corresponding to the levels of non-haem iron species associated with sickle membranes, was examined in this system after extensive dialysis against EDTA.

3. RESULTS AND DISCUSSION

To elucidate the catalytic properties of iron-containing components associated with the sickle erythrocyte membrane electron spin resonance spectroscopy was applied using the spin trap DMPO. The ability of the membrane-bound iron complexes retained by sickle erythrocyte membranes to initiate the breakdown of a model hydroperoxide, *t*-butyl hydroperoxide, to alkoxyl and peroxy radical species was assessed: typical ESR spectra of alkoxyl-DMPO and peroxy-DMPO radical adducts are shown in Fig. 1. Significantly higher concentrations of the alkoxyl and peroxy radical spin adducts were produced by sickle erythrocyte membranes compared to normal membranes (Table I).

The levels and availability for reaction at physiological pH of non-haem iron species associated with sickle membranes were assessed as described in section 2. One component of this iron reacted within 60 min in this assay. This available component was present at levels of 6.1 ± 4 nmol iron/mg membrane protein ($n = 13$ patients) which represents $46 \pm 11\%$ ($n = 13$) of the total non-haem iron associated with the erythrocyte membranes of this group of patients. The remainder of the iron species was unavailable to ferrozine within a realistic time scale. Non-haem iron was not detected in normal membrane preparations.

Investigations were undertaken to correlate the extent of the hydroperoxide breakdown, in terms of peroxy radical generation, with the levels of non-haem iron associated with erythrocyte membranes from different sickle patients. The initial rate of peroxy radical spin adduct generation from hydroperoxide catalysed by sickle erythrocyte membranes correlated ($r = 0.95$) (Fig. 2) with the levels of the more available component of the iron component.

The presence of a more available and less available component of non-haem iron associated with sickle erythrocyte membranes is consistent with earlier reports

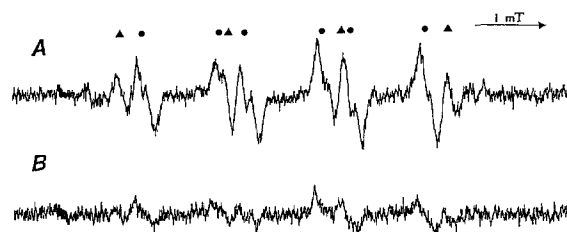


Fig. 1. ESR spectra observed on reaction of erythrocyte membranes with *t*-BuOOH in the presence of the spin trap DMPO. Spectra of incubations (22°C, air-saturated, pH 7.4) containing sickle cell erythrocyte membranes (A) or normal erythrocyte membranes (B), 10 mM *t*-BuOOH and 40 mM DMPO were recorded 90 s after mixing. Spectra assigned to a mixture of the peroxy (●) and alkoxyl (▲) radical adducts to DMPO by comparison of hyperfine coupling constants with previously reported data [23].

[8,9] in which it has been suggested that the less available component may represent iron within ferritin. In the present studies, ferritin released iron very slowly in the non-haem iron assay, comparably with the less available component of non-haem iron associated with sickle erythrocyte membranes, whereas ferrous and ferric iron salts reacted to completion within 1 min. Ferritin, at suitable concentrations, admixed with normal membranes or alone demonstrated no detectable catalytic activity on *t*-butyl hydroperoxide, as expected, when examined by ESR spectroscopy.

The effects of desferrioxamine on the production of alkoxyl and peroxy radicals in this system were studied by preincubating sickle or normal erythrocyte membranes with desferrioxamine (90 μ M) for 1 h at 22°C before the addition of *t*-butyl hydroperoxide (10 mM) and DMPO (40 mM). This treatment significantly reduced the levels of alkoxyl and peroxy radical spin adducts (Table I) and in addition signals from the

Table I

Radical generation from hydroperoxide catalysed by membrane-associated iron-species and the effect of desferrioxamine (DFO)

Treatment	Peroxy radical adduct signal (peak height in mm/unit membrane concentration)	
	Sickle erythrocyte membranes	Normal erythrocyte membranes
none	$7.93 \pm 1.6^*$	4.11 ± 1.3
dexferrioxamine (90 μ M)	$5.79 \pm 1.3^\dagger$	5.02 ± 1.7

Sickle and normal membranes were added to *t*-butyl hydroperoxide and the radical generation after 150 s was assessed by electron spin resonance spectroscopy using the spin trap DMPO. Identical membrane aliquots were pretreated with desferrioxamine before examination in the same way. Values are given as peak-to-peak line heights of the peroxy radical adduct in mm obtained using identical machine conditions, after division by the protein concentration of the membrane preparation in mg/ml. Values are mean $\pm$ SD for 5 determinations.

* Different from normal membranes ($P < 0.01$, two sample *t*-test)

† Different from untreated sickle membranes ($P < 0.01$, paired *t*-test)

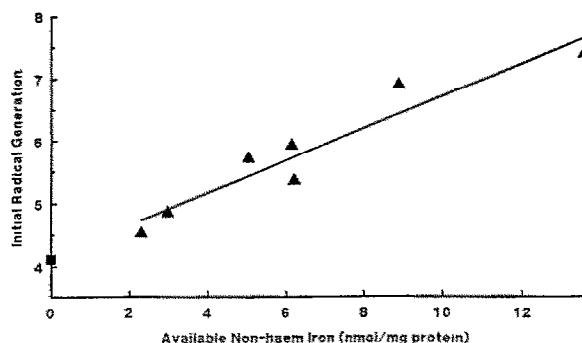


Fig. 2. Available non-haem iron-species associated with the sickle membrane and generation of radical species from hydroperoxide. The levels of non-haem iron reacting within 60 min in the ferrozine assay are shown for sickle erythrocyte membranes from different patients and correlated with the radical generation induced by these membranes as measured by electron spin resonance spectroscopy (▲, peroxy radical adduct measured after 150 s; see text). Radical generation from hydroperoxide in the same system induced by normal membranes (■, average of 5 preparations) is shown for comparison.

desferrioxamine nitroxide radical [14] were also detected (Fig. 3). These responses were more extensive at higher desferrioxamine concentrations, with greater suppression of the signals corresponding to alkoxy and peroxy radical adducts such that at 10 mM the signals were almost completely eliminated and greatly increased concentrations of desferrioxamine radicals were observed. In similar experiments incorporating ferrioxamine (the iron complex of desferrioxamine) no nitroxide signals were observed. No DMPO adducts to the hydroxyl radical were observed in these experiments.

When normal erythrocyte membranes were similarly preincubated with desferrioxamine at concentrations of 10 mM or greater, the levels of the alkoxy and peroxy DMPO radical adducts were diminished and the desferrioxamine nitroxide radical was observed. Lower concentrations of desferrioxamine (90 μ M) were ineffective. Breakdown of hydroperoxides by normal erythrocyte membranes is attributable to the low levels of associated haem iron. Similar results were obtained from experiments carried out to assess the scavenging of alkoxy and peroxy radicals by desferrioxamine in a model system utilising *t*-butyl hydroperoxide and methaemoglobin in the absence of non-haem iron (data not shown).

Apart from its capacity to chelate ferric iron, desferrioxamine can be oxidised by the superoxide radical [14], has been reported to act as an electron donor to the ferryl myoglobin radical [12], and has recently been shown to scavenge the peroxy radical in suspensions of linoleic acid in which oxidation has been initiated by a thermolabile azo compound [24]. In this study desferrioxamine has been shown to decrease the intensity of the observed alkoxy and peroxy DMPO radical adducts generated by endogenous iron complexes

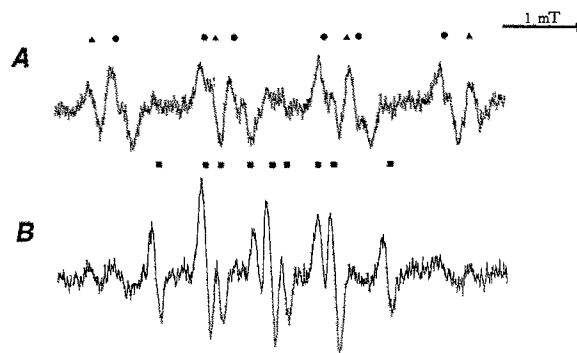


Fig. 3. Alkoxy-DMPO and peroxy-DMPO radical adduct signals as detected by ESR produced by the reaction of sickle erythrocyte membranes with *t*-butyl hydroperoxide with and without desferrioxamine. Spectra were recorded at pH 7.4 and 22°C, under air-saturated conditions, 400 s after mixing. Sickle erythrocyte membranes were mixed with *t*-butyl hydroperoxide (10 mM) and DMPO (40 mM) in the absence of desferrioxamine (A) and with 90 μ M desferrioxamine (B). Spectra assigned to a mixture of the peroxy (●) and alkoxy (▲) radical adducts to DMPO and to the desferrioxamine nitroxide radical (■).

associated with sickle erythrocyte membranes and by other systems.

The generation of the desferrioxamine nitroxide radical in all of these systems indicates that desferrioxamine is acting as a chain breaking radical scavenger by donating an electron or hydrogen atom from the hydroxamate centre, although the possibility that desferrioxamine is additionally involved in chelating available non-haem iron associated with the sickle erythrocyte membrane cannot be excluded. This may have important implications in the development of possible strategies to suppress the oxidative damage in sickle erythrocytes.

Acknowledgements: We thank Professor A. Bellingham for providing sickle erythrocyte samples. We acknowledge financial support from the Peter Samuel-Royal Free Fund and the Science and Engineering Research Council (Quota award to A.H. and ESR spectroscopy facilities at University of York).

REFERENCES

- [1] Keberle, H. (1964) *Anal. NY Acad. Sci.* 119, 758-764.
- [2] Asakura, T., Minakata, K., Adachi, K., Russel, M.O. and Schwartz, E. (1977) *J. Clin. Invest.* 59, 633-640.
- [3] Bauminger, E.R., Cohen, S.G., Ofer, S. and Rachmilewitz, E.A. (1979) *Proc. Natl. Acad. Sci. USA* 76, 939-943.
- [4] Rice-Evans, C., Omorphos, S.C. and Baysal, E. (1986) *Biochem. J.* 237, 265-269.
- [5] Rice-Evans, C. and Baysal, E. (1987) *Acta Haematol.* 78, 105-108.
- [6] Kuross, S.A., Rank, B.H. and Hebbel, R.P. (1988) *Blood* 71, 876-882.
- [7] Hartley, A. and Rice-Evans, C. (1989) *Biochem. Soc. Trans.* 17, 116-118.
- [8] Kuross, S.A. and Hebbel, R.P. (1988) *Blood* 72, 1278-1285.
- [9] Hartley, A. and Rice-Evans, C. (1989) *Biochem. Soc. Trans.* 17, 488-489.

- [10] Morehouse, K.M., Flitter, W.D. and Mason, R.P. (1987) *FEBS Lett.* 222, 246-250.
- [11] Kanner, J. and Harel, S. (1987) *Free Rad. Res. Commun.* 3, 309-317.
- [12] Rice-Evans, C., Okunade, G. and Khan, R. (1989) *Free Rad. Res. Commun.* 7, 45-54.
- [13] Rice-Evans, C. and Okunade, G. (1989) in: *Free Radicals, Diseased States and Anti-Radical Interventions* (Rice-Evans, C. ed.) pp. 389-412, Richelieu Press, London.
- [14] Davies, M.J., Donker, R., Dunster, C.A., Gee, C.A., Jonas, S. and Willson, R.L. (1987) *Biochem. J.* 246, 725-729.
- [15] Rice-Evans, C., Baysal, E., Flynn, D. and Kontoghiorghe, G. (1986) *Biochem. Soc. Trans.* 14, 368-369.
- [16] Rice-Evans, C., Baysal, E., Singh, S., Jones, S. and Jones, J.G. (1989) *FEBS Lett.* 256, 17-20.
- [17] Puppo, A. and Halliwell, B. (1988) *Planta* 173, 405-410.
- [18] Gutteridge, J.M.C. (1987) *FEBS Lett.* 246, 362-364.
- [19] Dodge, J.T., Mitchell, C. and Hanahan, D.J. (1963) *Arch. Biochem. Biophys.* 100, 119-128.
- [20] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) *J. Biol. Chem.* 193, 265-275.
- [21] Ceriotti, F. and Ceriotti, G. (1980) *Clin. Chem.* 26, 327-331.
- [22] Stookey, L.L. (1970) *Anal. Chem.* 42, 779-781.
- [23] Davies, M.J. (1988) *Biochem. Biophys. Acta* 964, 28-35.
- [24] Darley-Usmar, V.M., Hersey, A. and Garland, L.G. (1989) *Biochem. Pharmacol.* 38, 1465-1469.