Volume 256, number 1,2, 17–20 FEB 07669 October 1989

The interactions of desferrioxamine and hydroxypyridone compounds with haemoglobin and erythrocytes

Catherine Rice-Evans, Erol Baysal[+], Surrinder Singh[°], Shelley-Anne Jones* and J. Gareth Jones*

Department of Biochemistry, Royal Free Hospital School of Medicine, Rowland Hill Street, London NW3 2PF, England, [+]Department of Pharmacology, New York University, New York, USA, [°]Department of Pharmacy, King's College, University of London, London, England and **Department of Biochemistry, University College, Cardiff, Wales*

Received 3 August 1989

The iron chelator desferrioxamine has been applied in many studies of pathological states by several investigators. The resulting decreases in cellular and tissue damage have been interpreted as an indication of the involvement of iron-mediated radical species. Although the nature and location of the iron species have not been identified, the assumption has often been made that desferrioxamine is able to enter cells. This paper reports investigations of the ability of desferrioxamine to cross the erythrocyte membrane in comparison with that of specific hydroxypyridone iron chelators by assessing their interaction with haemoglobin.

Hydroxypyridone; Desferrioxamine; Haemoglobin; Iron chelator; Erythrocyte membrane

1. INTRODUCTION

Desferrioxamine is currently the only iron chelator in therapeutic use for the treatment of pathological disorders involving iron excess and iron decompartmentalisation. However, this chelator is not entirely non-toxic at standard or high doses [1–5] during prolonged clinical use unless the dosage regimen is closely adjusted to the iron status of the patient. In particular, cerebral and ocular toxicity have been reported in some patients (reviewed in [6]).

In vitro studies have proposed that the iron-binding site of desferrioxamine has an oxidising effect when in direct interaction with haemoglobin released from erythrocytes [7,8]; this may relate to the ability of the drug to interact with the superoxide radical [9–11] resulting in the formation of methaemoglobin. Other studies have demonstrated the ability of desferrioxamine to act as an electron donor [12,13]. Its ability to suppress the propagation of membrane lipid peroxidation [13–15] and to act as a peroxyl radical scavenger [16] has also been reported.

This paper reports investigations of the ability of desferrioxamine to cross the membrane of the erythrocyte in comparison with that of specific hydroxypyridone iron chelators selected on the basis of their varying capacities for traversing cell membranes [17]. The responses were assessed of (i) haemoglobin within the erythrocyte to the iron chelators presented extracellularly, in the presence and absence of iron-mediated oxidative stress, and of (ii) haemoglobin, released from erythrocytes, and directly exposed to the drugs. The results suggest that desferrioxamine does not enter the erythrocyte within a timescale longer than its half-life in plasma.

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

[+] This is a present address

2. EXPERIMENTAL

Fresh human erythrocytes were obtained from normal, healthy donors and used immediately. After centrifugation, the plasma and buffy coat were removed and the erythrocytes

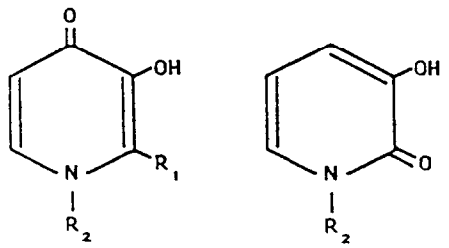
Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

washed three times with iso-osmotic phosphate buffer, pH 7.4. Erythrocytes at a 5% suspension in iso-osmotic buffer were incubated at 37°C for various time intervals up to 24 h. For haemolysis the original erythrocytes were lysed in 20 vols of ice-cold hypo-osmotic buffer, pH 7.4, the membranes removed and treated as in the intact cell experiments. Experiments employing a range of proportions of volumes of the hypo-osmotic haemolysis buffer, down to 2 vols, were performed such that the final haemoglobin to chelator ratio was as close as possible to that which would be attainable assuming total uptake by the erythrocytes of the desferrioxamine.

The specific model of iron-mediated oxidative stress consisted of a iron(II)/ascorbate/hydrogen peroxide stress system [18,19] presented extracellularly to erythrocytes at levels such as to induce intracellular iron stress in the form of iron-containing inclusion bodies from denatured haemoglobin, akin to the situation in thalassaemia major [20]. The experimental systems consisted of iron(II) sulphate (100 μM), ascorbic acid (1 mM), hydrogen peroxide (200 μM). Iron chelators were utilised at concentrations such that desferrioxamine was in an appropriate ratio to haemoglobin as that observed in situations of microbleeding and in a 4-fold excess of the exogenously added iron (400 μM), giving the same ratio as that in the clinical treatment of iron overload. Iron chelators were added prior to the iron stress in all cases. Specific hydroxypyridone iron chelators, which coordinate iron in a 3:1 ratio (structures in fig.1), were selected on the basis of their relative abilities to traverse cell membranes [17]: CP02 crosses the membrane (K_{part} 0.5 for octanol/Tris buffer 20 mM), CP22 will enter the membrane readily (K_{part} 1.35), whereas CP47 does not enter the membrane (K_{part} <0.002). Final concentrations of the hydroxypyridone chelators (1 mM) were estimated on the basis of their stoichiometry of binding of iron compared to the equimolar stoichiometry of desferrioxamine.

Erythrocyte filterability was assessed by filtering suspensions (30×10^6 cells/ml) through 3 μm Nucleopore membranes. Erythrocyte transit time (Torr) was then calculated by analysis of complete flow profiles from zero to 1 min [21].

Methaemoglobin formation was measured by the decrease in absorbance at 620 nm after the addition of cyanide [22].



Pyridin-4-one Pyridin-2-one

Fig.1. The structures of hydroxy pyridin-2-one and hydroxy pyridin-4-one compounds. 3-Hydroxy pyridin-4-one, R_1 = $-CH_3$; R_2 = $-CH_2CH_2CH_3$, CP22; R_2 = $-(CH_2)_4CH(NH_3^+)COO^+$, CP47. 3-Hydroxy pyridin-2-one, R_2 = $-CH_2CH_3$, CP02.

3. RESULTS AND DISCUSSION

The effects of desferrioxamine and ferrioxamine on the filterability of erythrocytes under iron-mediated oxidative stress are shown in table 1. Filterability is expressed in terms of transit time. Exposure of normal erythrocytes extracellularly to the iron stress system for 4 h revealed a 2.5-fold increase in the transit time of the cells (table 1). When desferrioxamine was incorporated into the incubation, prior to the iron stress system, to suppress the generation of oxygen radicals extracellularly by chelating the iron, the diminished filterability (increased transit time) was not modified. Control incubations of erythrocytes with ferrioxamine or desferrioxamine in the presence of peroxide and ascorbate show that although ferrioxamine does not alter the filterability, desferrioxamine does increase the pore transit time by more than 30% after 4 h, which cannot be accounted for by the effects of ascorbate and hydrogen peroxide on the erythrocytes. This result may imply a weak interaction of desferrioxamine at the membrane surface.

The effectiveness of the hydroxypyridone compounds in the inhibition of intracellular haemoglobin oxidation induced by the extracellular iron-mediated oxidant stress system in comparison with desferrioxamine, is shown in table 2. CP22, CP47 and desferrioxamine were the

Table 1

The effects of desferrioxamine and ferrioxamine on the filterability of erythrocytes under iron-mediated oxidative stress

Additions	Transit time (Torr)
None	0.67 ± 0.18 (14)
Iron/ascorbate/hydrogen peroxide	1.67 ± 0.38 (10)
Desferrioxamine + iron stress	1.37 ± 0.47 (6)
Ferrioxamine + iron stress	1.81 ± 0.44 (4)
Desferrioxamine + ascorbate/hydrogen peroxide	1.02 ± 0.18 (4)
Ferrioxamine + ascorbate/hydrogen peroxide	0.63 ± 0.16 (4)

Filterability is expressed in terms of transit time. Cell suspensions contained 30×10^6 cells/ml

Table 2

The effects of hydroxypyridones and desferrioxamine on intracellular haemoglobin oxidation induced by extracellular iron-mediated oxidative stress

Additions	% methaemoglobin	
	5 h	24 h
None	2 ± 1	16 ± 1
Iron stress	11 ± 4	85 ± 7
Desferrioxamine (0.4 mM) + iron stress	2 ± 1	40 ± 5
CP02 (1 mM) + iron stress	30 ± 5	68 ± 7
CP22 (1 mM) + iron stress	4.8 ± 3	40 ± 4
CP47 (1 mM) + iron stress	3.1 ± 2	38 ± 5
	$n = 4$	

most effective in suppressing iron-induced methaemoglobin production, at 5 h of incubation, compared to the extent of haemoglobin oxidation in their absence, whereas CP02 responded differently, enhancing haemoglobin oxidation. After prolonged incubation for 24 h, CP22, CP47 and desferrioxamine decreased the intracellular haemoglobin oxidation induced by extracellular exposure to the iron stress system to approximately the same extent. CP02 was again less effective.

In order to clarify the effects of the chelators in the absence of the iron stress system, control erythrocytes were treated with the iron chelators alone and the response assessed in terms of methaemoglobin production (table 3). None of the erythrocyte incubations showed any variation in response from that of the control in the absence of the chelators except for CP02 which enhanced the oxidation of haemoglobin. This chelator is the one of the selected hydroxypyridones which crosses the membrane readily and the enhanced methaemoglobin production can be explained by the direct interaction with haemoglobin on entering the cell.

To determine the reactivity of the compounds with haemoglobin their direct exposure to red cell haemolysates was investigated. Table 3 also indicates that the response of haemoglobin in membrane-free haemolysates to direct interaction with the drugs in terms of the extent of haemoglobin oxidation is much enhanced in the presence of CP02 and desferrioxamine but this toxic response is not elicited by CP22 and CP47.

The relative abilities of the iron chelators to induce haemoglobin oxidation and the responses observed when the compounds are presented to the intact cells or directly to the haemolysate suggest that desferrioxamine does not cross the erythrocyte membrane under these conditions and does not enter the erythrocytes within a timescale longer than its half-life in plasma. Desferrioxamine has a low lipid solubility, K_{part} 0.01 [17], and it is possible that it is this characteristic of the drug which limits its ability to penetrate most cells of the body [6].

The iron chelator desferrioxamine has been applied by several investigators in studies in vitro of pathological states in which excess superoxide generation has been implicated; a resulting decrease in tissue damage has led to the conclusion

Table 3

The effects of the hydroxypyridones and desferrioxamine on haemoglobin by direct and by extracellular exposure

Additions	% methaemoglobin				
	Erythrocytes		Haemolysate		
	5 h	24 h	5 h	10 h	24 h
None	0	16 ± 1	2.6 ± 1	5.7 ± 2	14 ± 3
Desferrioxamine					
0.4 mM	0	16 ± 1	14.2 ± 3	28.0 ± 5	53.5 ± 8
0.1 mM	0	16 ± 1	10.0 ± 2	13.0 ± 2	30.0 ± 1
CP02 (1 mM)	2.0 ± 1	25 ± 5	4.2 ± 2		35 ± 8
CP22 (1 mM)	0	14 ± 3	3.0 ± 1	6.0 ± 2	11.5 ± 2
CP47 (1 mM)	0	14 ± 2	3.0 ± 1	5.5 ± 1	11.7 ± 3
	$n = 4$		$n = 6$		

that hydroxyl radicals or other reactive species involving iron-oxygen complexes may have been involved. Although the nature and location of the iron species have not been identified in many instances, the assumption has often been made that desferrioxamine is able to enter the cells in question, for which the evidence is very limited. For example, the ability of desferrioxamine to clear iron from iron-loaded hepatocytes may be mediated by a mechanism of uptake specific to hepatocytes [23]. The iron from these cells would eventually be excreted in the bile, while the source of iron excreted in the urine may all be extracellular chelation, a mechanism which would include iron release from reticuloendothelial cells [24].

Acknowledgement: Financial assistance from the Turkish Government of Northern Cyprus is acknowledged.

REFERENCES

[1] Davies, S.E., Marcus, R.E., Hungerford, J.L., Miller, M.H., Arden, G.B. and Huehns, E.R. (1983) Lancet ii, 181–184.
[2] Blake, D.R., Winyard, P., Lunec, J., Williams, A., Good, P.A., Crewes, S.J., Gutteridge, J.M., Rowley, D., Halliwell, B., Cornish, A. and Hider, R.C. (1985) Q. J. Med. 56, 345–355.
[3] Lakhanpal, V., Shocket, S.S. and Jiji, R. (1984) Ophthalmology 91, 443–451.
[4] Guerin, A., London, G., Marchais, S., Metivier, F. and Pelisse, J.M. (1985) Lancet ii, 39–40.
[5] Olivieri, N.F., Buncic, J.R., Chew, E., Gallant, T., Harrison, J.V., Keenan, N., Logan, W., Mitchell, D., Ricci, G., Skarf, B., Taylor, M. and Freedman, N.H. (1986) New Engl. J. Med. 314, 869–873.
[6] Porter, J.B. and Huehns, E.R. (1989) in: Bailliere's Clinical Haematology, Iron Chelating Therapy, vol.2, no.2, pp.459–474, Bailliere Tindall, London.
[7] Rice-Evans, C., Baysal, E., Flynn, D. and Kontoghiorghes, G. (1986) in: Superoxide and Superoxide Dismutase in Chemistry, Biology and Medicine (Rotilio, G. ed.) pp.162–166, Elsevier, Amsterdam.
[8] Rice-Evans, C., Baysal, E., Flynn, D. and Kontoghiorghes, G. (1986) Biochem. Soc. Trans. 14, 368–369.
[9] Halliwell, B. (1985) Biochem. Pharmacol. 34, 229–233.
[10] Davies, M.J., Donkor, R., Dunster, C.A., Gee, C.A., Jonas, S. and Willson, R.L. (1987) Biochem. J. 246, 725–729.
[11] Morehouse, K.M., Flitter, W.D. and Mason, R.P. (1987) FEBS Lett. 222, 246–250.
[12] Kanner, J. and Harel, S. (1987) Free Rad. Res. Commun. 3, 309–317.
[13] Rice-Evans, C., Okunade, G. and Khan, R. (1989) Free Rad. Res. Commun., in press.
[14] Rice-Evans, C., Baysal, E. and Omorphos, S.C. (1986) Biochem. J. 237, 265–269.
[15] Hartley, A., Rice-Evans, C. and Davies, M.J. (1989) submitted.
[16] Darley-Usmar, V.M., Hersey, A. and Garland, L. (1989) in: Free Radicals, Diseased States and Antiradical Interventions (Rice-Evans, C. ed.) Richelieu Press, London, in press.
[17] Porter, J., Gyparaki, G., Burke, L.C., Huehns, E.R., Sarpong, P., Saez, V. and Hider, R.C. (1988) Blood 72, 1497–1503.
[18] Rice-Evans, C. and Baysal, E. (1987) Biochem. J. 244, 191–196.
[19] Rice-Evans, C. (1987) in: Free Radicals, Oxidant Stress and Drug Action (Rice-Evans, C. ed.) pp.307–330, Richelieu Press, London.
[20] Rachmilewitz, E.A. (1985) Clin. Haematol. 14, 163–182.
[21] Jones, S.-A., Dempsey, T., Jones, J.G. and Rice-Evans, C. (1988) Biochem. Soc. Trans. 16, 292–293.
[22] Harley, J.O. and Mauer, A.M. (1960) Blood 16, 1722–1735.
[23] Porter, J.B., Burke, L.C., Pippard, M.J., Hider, R.C. and Huehns, E.R. (1987) Abstract, British Society for Haematology, Lancaster, October 1987.
[24] Hershko, C., Grady, R.W. and Cerami, A. (1978) J. Clin. Lab. Med. 92, 144–149.