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THIOL COMPOUNDS AS PROTECTIVE AGENTS IN ERYTHROCYTES UNDER OXIDATIVE STRESS

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The potential for the thiol-containing drugs, N-acetyl cysteine and N-mercaptopropionyl glycine, to act as antioxidants intracellularly has been studied in erythrocytes under oxidative stress. The effects have been compared with that of the glutathione peroxidase inhibitor, mercaptosuccinate. The results show differential responses of sickle and normal erythrocytes to the thiol compounds. N-acetyl cysteine is the more efficacious with no toxic effects in these systems. N-Mercaptopropionyl glycine is not only limited in its ability to demonstrate antioxidant capacity in erythrocytes but also exerts deleterious effects.

KEY WORDS: sickle erythrocyte, antioxidant, membrane, N-acetyl cysteine, mercaptosuccinate, mercaptopropionyl glycine.

INTRODUCTION

Compounds containing a thiol group, such as N-acetyl cysteine (NAC), 2-mercaptopropionyl glycine (MPG), are known for their efficacy in restoring the activity of protein-bound sulphydryl groups. The ability of NAC to protect cells and tissues (and particularly studied has been the lung) against oxidative damage has been attributed to radical scavenging and to the enhancement of the availability of cysteine, a substrate for glutathione synthesis.[1] NAC is reported to be a scavenger of hydroxyl radicals[2] and ferryl myoglobin radical species[3] as well as displaying a ready reactivity with hypochlorous acid,[2] whereas its reaction with hydrogen peroxide is slow. MPG has been used in the therapy of heavy metal poisoning and haemochromatosis.[4] It is also recognised as a scavenger of ferryl myoglobin radicals and hydroxyl radicals.[3,5] Recent studies have shown MPG to be effective in suppressing myocardial reperfusion injury in *in vivo* animal models.[5] Mercaptosuccinate is a thiol-containing inhibitor of the selenium-dependent enzyme, glutathione peroxidase, a property which MPG has been reported to exhibit, to a lesser extent.[6]

Markers of free radical-mediated damage in sickle erythrocytes have been recognised for a number of years. Reduced glutathione levels are diminished on average by 20%.[7] Increased endogenous levels of lipid peroxides and their secondary metabolites have been identified in sickle erythrocyte membranes.[8-12] In particular, samples

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containing higher proportions of irreversibly sickled cells retain elevated levels of membrane-associated iron[13,14] which has been implicated in the potentiation of peroxidative membrane damage.[15] *In vitro* studies have demonstrated the suppression of membrane damage on incorporation of peroxyl radical scavengers which act as chain-breaking antioxidants.[9,12]

We have examined the ability of thiol-containing compounds to (i) modulate oxidative stress in sickle erythrocytes and (ii) attenuate oxidative damage in sickle erythrocytes compared with normal erythrocytes applying exogenous oxidative stress which preferentially initially consumes intracellular reduced glutathione. The results show that N-acetyl cysteine is highly effective as an antioxidant in sickle erythrocytes in *in vitro* systems, in contrast with N-mercaptopropionyl glycine which demonstrates toxic properties.

MATERIALS AND METHODS

All chemicals used were of analytical grade. N-acetyl cysteine (NAC), mercaptopropionyl glycine (MPG) were obtained from Sigma Chemical Company. Heparinised blood from normal donors and patients with sickle cell anaemia was taken and used within 16 hours. The red cells were separated and washed three times with isotonic phosphate buffer 5 mM (pH 7.4). The washed erythrocytes were incubated at 2.5% haematocrit for 1 h at 37°C with NAC, MPG and mercaptosuccinate, 1 mM concentration. Exogenous oxidative stress was applied in the form of 0.1 mM *t*-butyl hydroperoxide at 37°C for 1 h, conditions which did not induce lipid peroxidation in the normal erythyrocyte membranes. After incubation, the cells were washed twice with isotonic phosphate buffer. The reduced GSH levels of red blood cells were determined spectrophotometrically applying 5,5′dithiobis(2-nitrobenzoic acid) at 412 nm.[16] Normal and sickle erythrocyte membranes were prepared by hypotonic haemolysis.[17] The membrane protein concentration was determined by the Lowry assay,[18] using bovine serum albumin as a standard. Membrane lipid peroxidation was assayed using the modified thiobarbituric acid assay[19] with the absorbance of the chromophore, measured at 532 nm, corrected for the background absorbance at 580 nm due to possible contributions from haem proteins. Appropriate controls were incorporated according to Gutteridge *et al.* [20] Free thiol levels of membrane proteins were analysed by the spectrophotometric method of Haest *et al.*[21] using reduced glutathione as standard.

RESULTS

The intracellular thiol levels of red blood cells obtained from healthy donors and sickle patients was determined by measuring the reduced glutathione level. Consistent with earlier reports,[22,23] the reduced GSH level of sickle erythrocytes from patients in this study showed a 25% decrease ($p < 0.001$) when compared to the normals. Treatment of normal erythrocytes with the thiol-containing drugs N-acetyl cysteine, N-mercaptopropionyl glycine and mercaptosuccinate markedly enhanced the intracellular thiol level in these cells. When sickle cells were exposed to the thiol compounds, under the same conditions, on the other hand, no significant modification in the intracellular thiol levels was detected for any of the drugs (Figure 1).

Normal and sickle erythrocytes were exposed to exogenous oxidative stress by

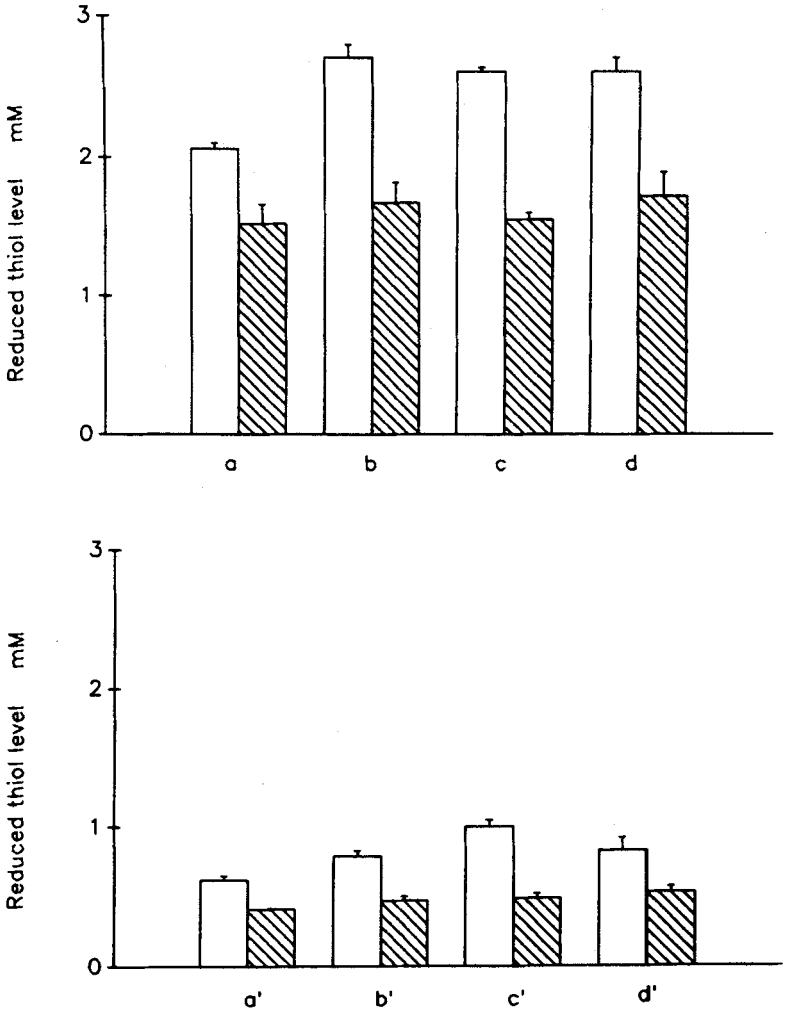

FIGURE 1 Intracellular thiol levels in control and sickle erythrocytes treated with N-acetyl cysteine (NAC), mercaptopropionyl glycine (MPG) and mercaptosuccinate (MS) (all at 1 mM, 1 hr). *Upper panel* (a) control normal and sickle erythrocytes, (b) NAC-treatment, (c) MS-treatment, (d) MPG-treatment. Values are mean ± SD (n = 5). *Lower panel* (a′) t-butyl hydroperoxide stressed normal and sickle erythrocytes, (b′) NAC-treatment, (c′) MS-treatment, (d′) MPG-treatment.

treating with 0.1 mM t-butyl hydroperoxide for 1 h (a condition which did not induce membrane lipid peroxidation in normal red cells). t-Butyl hydroperoxide preferentially reacts intracellularly with the reduced glutathione prior to initiating haemoglobin oxidation and membrane lipid peroxidation.[24] The time-scale was selected after studying the effects of 0.1 mM hydroperoxide at varying times of incubation on the reduced glutathione levels (Figure 2). The results indicate that initially the reduced glutathione levels are depressed to 0.4 mM but subsequently recover and stabilise at 0.6 mM after 1–3 h after addition of the hydroperoxide. The protective effects exerted

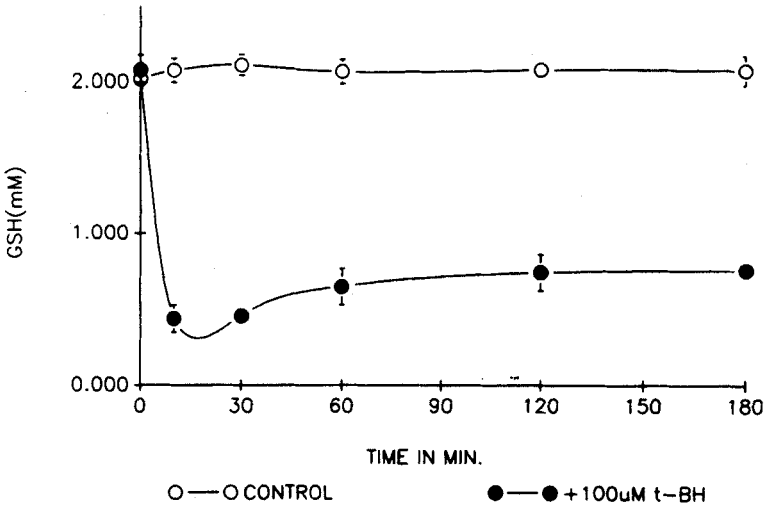

FIGURE 2 The time dependence of intracellular glutathione levels in normal erythrocytes on exposure to *t*-butyl hydroperoxide, O control incubation, ● *t*-butyl hydroperoxide treatment (0.1 mM). Values are mean ± SD (n = 5).

by the thiol compounds on the erythrocytes against the effects of *t*-butyl hydroperoxide-induced stress are depicted in Figure 1. *t*-Butyl hydroperoxide-induced oxidative stress (0.1 mM) caused pronounced depletion (*ca.* 75%) of reduced GSH. Prior incubation with thiol compounds protected the normal erythrocytes against thiol depletion significantly. Among the three, mercaptosuccinate was more protective against loss of the thiol group implying its ability to cross the membrane more readily than NAC or MPG. However, none of the compounds exercised a significant protection of the sickle erythrocytes from *t*BH-induced oxidative stress.

To investigate membrane alterations, lipid peroxidation and membrane protein thiol levels were measured. Red blood cell membranes were prepared after incubating intact cells (sickle and normal) with thiol compounds with and without oxidative stress. Exposure of normal red blood cells to 0.1 mM hydroperoxide for 1 h induced no peroxidation in the membrane lipids in our study and, thus, the presence of thiol compounds in the incubating system manifested no change. *t*-Butyl hydroperoxide at the same concentration, on the other hand, induced a marked elevation of thiobarbituric acid-reactive compounds in the sickle erythrocytes membranes (Figure 3). It was interesting to note that only NAC at 1 mM concentration attenuated the membrane lipid peroxidation and this antioxidant property was demonstrated in sickle erythrocytes both with and without exogenous oxidative stress.

Measurement of free thiol groups of membrane proteins was carried out as marker of oxidative protein damage in normal and sickle erythrocyte membranes after treatment of the erythrocytes with the thiol compounds in the presence and absence of *t*-butyl hydroperoxide-induced oxidative stress. Figure 4 illustrates pronounced oxidation of the thiol groups of normal erythrocyte membrane proteins in cells under exogenous oxidative stress. The membrane protein thiol oxidation observed in control normal erythrocytes under oxidative stress could be substantially suppressed by prior treatment of the cells with mercaptosuccinate, whereas the NAC and MPG were

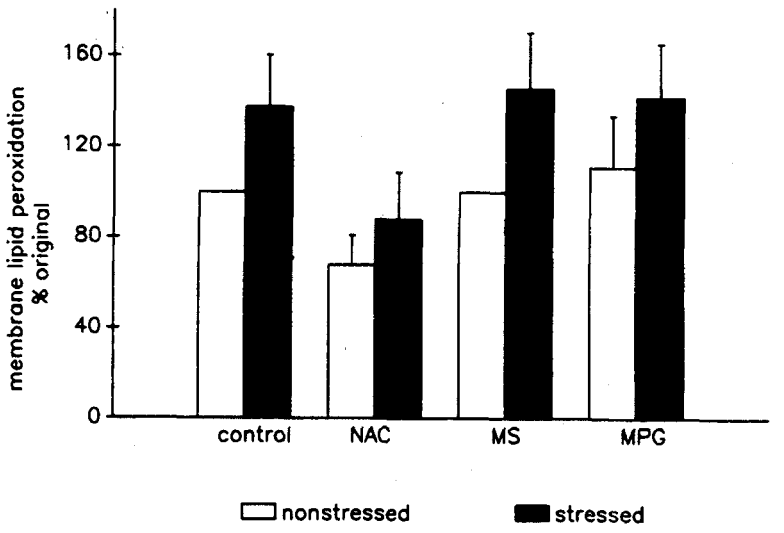

FIGURE 3 The effects of the thiol compounds in suppressing *t*-butyl-hydroperoxide-induced lipid peroxidation in sickle erythrocyte membranes in the presence of NAC, in the presence of MS, and in the presence of MPG [1 mM drugs, 0.1 mM *t*-butyl hydroperoxide, 1 hr incubation]. Values are mean ± SD (n = 5).

ineffective. On the other hand, the extent of the membrane thiol oxidation in sickle erythrocytes under exogenous oxidative stress is unresponsive to all the thiol compounds, whereas, as mentioned above, NAC attenuated lipid peroxidation in these cells. In the absence of exogenous oxidative stress, however, MPG and MS enhance the membrane thiol oxidation in sickle erythrocytes.

A positive relationship exists between inhibition of protein thiol oxidation and enhancement of –SH equivalents within intact normal cells in the presence of mercaptosuccinate (see Figure 2(b) for comparison), which confirms the antioxidant role of thiol compounds in cells, especially under conditions of exogenous oxidative stress. On the other hand, the membrane protein thiol level in sickle cells remains unaltered and the thiol compounds exert no protection against oxidative stress.

DISCUSSION

Many studies have suggested that thiol-containing drugs may be important in protecting cells and tissues intracellularly from oxidative stress.[2] We are interested in evaluating the efficacies of N-acetyl cysteine and mercaptopropionylglycine as antioxidant drugs in pathological erythrocytes under oxidative stress compared to normal erythrocytes, and their response to exogenous oxidative stress selected at a level such that no peroxidation is induced in normal erythrocyte membranes. In addition, the membrane-penetrating thiol compound, the glutathione peroxidase inhibitor mercaptosuccinate is applied for comparison. The work described in this paper shows that, although NAC, MPG and mercaptosuccinate are equally effective in enhancing the intracellular thiol levels of normal erythrocytes, they exert no apparent effect on this parameter in sickle erythrocytes under the same conditions.

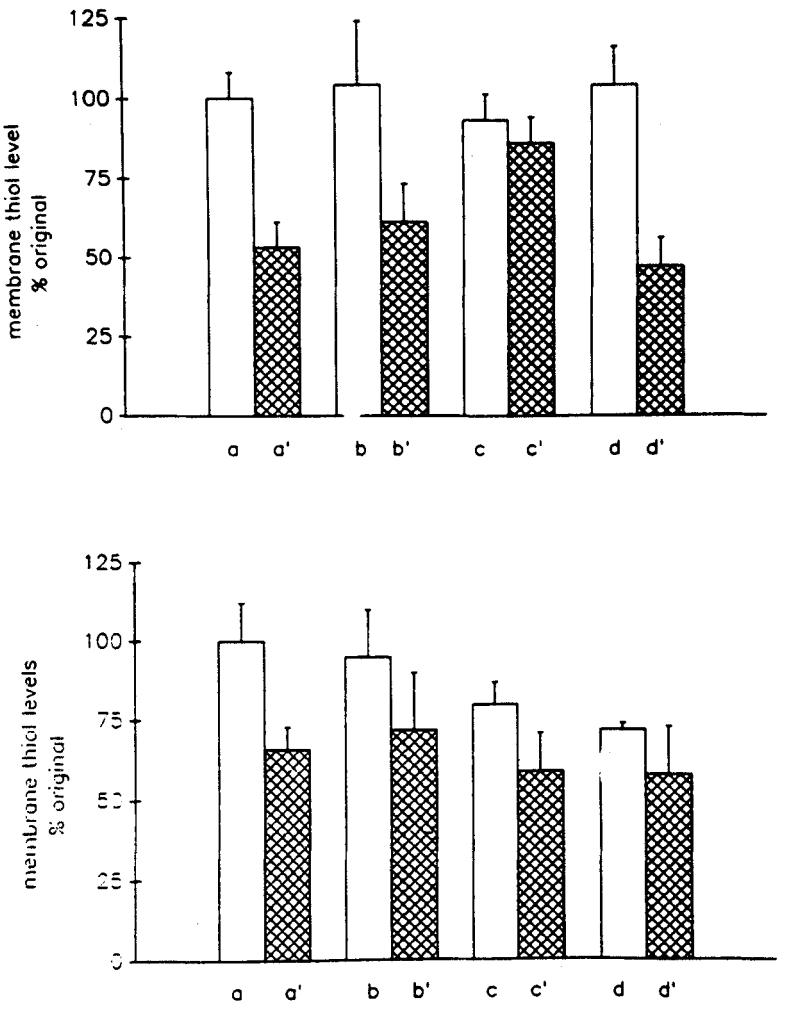

FIGURE 4 The effects of thiol compounds in protecting the membrane proteins from *t*-butyl hydroperoxide-induced oxidative stress. Upper: normal erythrocyte membranes unstressed and stressed, a, a′ unstressed and stressed, b, b′ NAC-treatment, unstressed and stressed, c, c′ MS-treatment, unstressed and stressed, d, d′ MPG-treatment, unstressed and stressed. Lower: sickle erythrocyte membranes unstressed and stressed. Columns as above. Values are mean ± SD (n = 5).

NAC is a known promoter of GSH synthesis *in vivo*, providing the critical precursor, cysteine, thus enhancing thiol levels in erythrocytes treated with this compound. MPG and mercaptosuccinate are capable of elevating the reduced GSH level in normal red cells by inhibiting glutathione peroxidase,[6] the latter being the major pathway for GSH consumption in tissues. The lack of response of the intracellular thiol levels in sickle erythrocytes to these compounds suggests either a slower access intracellularly via the sickle membrane or increased oxidation of these compounds upon entering the cells. However, the membrane data preclude the former consideration.

The drugs partially reversed the loss of intracellular thiol levels in normal erythrocytes exposed to exogenous oxidative stress (at a level that does not induce lipid peroxidation), mercaptosuccinate providing 50% protection and NAC and MPG providing 35% protection. Again in sickle erythrocytes under oxidative stress the thiol drugs elicited no response in the glutathione levels or total intracellular thiol levels.

N-acetyl cysteine is the most effective antioxidant against membrane damage. The protective property of NAC on lipid peroxidation is consistent with earlier reports of inhibition of incubation-induced propagation of oxidative damage in sickle membranes by antioxidants such as ascorbate, α-tocopherol and the trihydroxamate hydrogen-donor, desferrioxamine.[9] The mechanism of action of these compounds has been explained by their chain-breaking antioxidant property. NAC may be intercepting the propagation phase of peroxidising lipids in sickle membranes (with the concomitant formation of thiyl radical which may be removed by, for example, bimolecular termination). Identification of thiyl radical species by EPR, formed in other systems on the addition of NAC, without any subsequent deleterious effects has been reported earlier.[3] MPG and MS do not exhibit the same antioxidant properties in these erythrocyte systems. NAC exerts no action on the membrane thiol oxidation status, but intercepts the propagation of lipid peroxidation. For membranes prepared from normal erythrocytes after exposure to oxidative stress, MS and NAC exert a degree of protection but MPG amplifies the oxidative stress. The data suggest that the interaction of MPG with haemoglobin may induce formation of an intermediate enhancing oxidative stress rather than MPG acting directly as an antioxidant.

We have previously reported that MPG only has a limited inhibitory effect on erythrocyte membrane lipid peroxidation induced by ferryl myoglobin radical species compared with NAC and that MPG is capable of inducing lipid peroxidation in erythrocyte membranes in the presence of myoglobin.[3] Other studies of Puppo *et al.*[25] suggest that MPG is able to enhance iron release from the haem pocket of myoglobin in the presence of a large excess of hydrogen peroxide, whereas previous studies of Rice-Evans *et al.*[26] have shown that other reducing agents such as ascorbate or desferrioxamine decrease the propensity for iron release from myoglobin activated by hydrogen peroxide by reducing ferryl myoglobin to metmyoglobin. MPG, however, induced peroxidation of erythyrocyte membrane lipids in the presence of metmyoglobin (in the absence of hydrogen peroxide).

These studies again suggest that MPG, in contrast with NAC, is capable of interfering with the haem pocket in some way. These studies show that N-acetyl cysteine has a beneficial effect on sickle erythrocytes in inhibiting endogenous lipid peroxidation, as well as suppressing lipid peroxidation when exogenous oxidative stress is applied. However, the intracellular thiol level is unchanged, perhaps reflecting the oxidation of the drugs within the sickle cells and the limited ability of the glutathione-generating system to function as effectively once the exogenous oxidative stress is consumed. The intracellular thiol level in normal erythyrocytes is enhanced by all the thiol compounds under the conditions applied. The observations concerning these membrane permeant reductants are particularly important in view of the significance recently attached to the role of the membrane bound nonhaem iron in enhancing peroxidative damage to the sickle cell membrane. For example, Hebbel *et al.*[7] have recently implicated ascorbate in enhancing peroxidation in membranes of sickle erythrocytes by its efficacy as a reducing agent, although it is not clear what concentrations were applied.

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References

1. I. Cotgreave, P. Moldeus and I. Schuppe (1991) The metabolism of N-acetylcysteine by human endothelial cells. *Biochemical Pharmacology*, **42**, 13–16.
2. O.I. Aruoma, B. Halliwell, B.M. Hoey and J. Butler (1989) The antioxidant action of N-acetylcysteine: its reaction with hydrogen peroxide. *Free Radical Biology and Medicine*, **6**, 593–597.
3. J.J.O. Turner, C. Rice-Evans, M.J. Davies and E.S.R. Newman (1991) The formation of free radicals by cardiac myocytes under oxidative stress and the effects of electron-donating drugs. *Biochemical Journal*, **277**, 833–837.
4. G. Zimmer and J. Evers (1988) 2-Mercaptopropionyl glycine improves aortic flow after reoxygenation in working rat hearts. *Basic Research in Cardiology*, **83**, 445–451.
5. R. Bolli, M.O. Jeroudi, B.S. Patel, O.I. Aruoma, B. Halliwell, E.K. Lau and B.P. McCay (1989) Marked reduction of free radical generation and contractile dysfunction of antioxidant therapy begun at the time of reperfusion. Evidence that myocardial stunning is a manifestation of reperfusion injury. *Circulation Research*, **65**, 607–622.
6. J. Chaudiere, E.C. Wihelmensen and A.L. Tappel (1984) Mechanism of selenium glutathione peroxidase and its inhibition by mercaptocarboxylic acids and other mercaptans. *Journal of Biological Chemistry*, **259**, 1043–1050.
7. R.P. Hebbel (1990) The sickle erythrocyte in double jeopardy: autoxidation and iron decompartmentalisation. *Seminars in Haematology*, **27**, 51–69.
8. C. Rice-Evans, S.C. Omorphos and J. White (1982) The effects of hydrazine on erythrocytes. *Biochimica Biophysica Acta*, **691**, 367–371.
9. C. Rice-Evans, S.C. Omorphos and E. Baysal (1986) Sickle cell membranes and oxidative damage. *Biochemical Journal*, **237**, 265–267.
10. R.P. Hebbel, J.W. Eaton and M. Balasingam (1982) Spontaneous oxygen radical generation by sickle erythrocytes. *Journal of Clinical Investigation*, **70**, 1253–1259.
11. R.P. Hebbel and J.W. Eaton (1989) Pathology of heme interaction with the erythrocyte membrane. *Seminars in Haematology*, **26**, 136–140.
12. A. Hartley, M. Davies and C. Rice-Evans (1990) Desferrioxamine as a lipid chainbreaking antioxidant in sickle erythrocyte membranes. *FEBS Letters*, **264**, 145–148.
13. S.A. Kuross and R.P. Hebbel (1988) Non-heme iron in sickle erythrocyte membranes: association with phospholipids and potential role in lipid peroxidation. *Blood*, **72**, 1278–1285.
14. A. Hartley and C. Rice-Evans (1989) The nature of membrane-bound iron species involved in radical-mediated damage in sickle erythrocytes. *Biochemical Society Transactions*, **17**, 488–489.
15. C. Rice-Evans (1990) Iron-mediated oxidative stress in erythrocytes in J.R. Harris (ed.), *Blood cell Biochemistry*, pp. 429–453. Plenum Press, New York and London.
16. E. Beutler, D. Duron and B.M. Kelly (1963) Improved method for the determination of blood glutathione. *Journal of Laboratory and Clinical Medicine*, **61**, 882–886.
17. J.T. Dodge, C. Mitchell and D.J. Hanhanan (1963) The preparation and chemical characteristics of haemoglobin-free ghosts of human erythrocytes. *Archives of Biochemistry and Biophysics*, **100**, 119–128.
18. O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall (1951) Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry*, **193**, 265–275.
19. R. Walls, K.S. Kumar and P. Hochstein (1976) Aging human erythrocytes. Differential sensitivity of young and old erythrocytes to haemolysis induced by peroxide in the presence of thyroxine. *Archives of Biochemistry and Biophysics*, **174**, 463–468.
20. J.M.C. Gutteridge (1986) Aspects to consider when detecting and measuring lipid peroxidation. *Free Radical Research Communications*, **1**, 173–184.
21. C. Haest, G. Plasa, P. Kays and B. Deuticke (1978) Spectrin as a stabiliser of phospholipid asymmetry in the human erythrocyte membrane. *Biochimica Biophysica Acta*, **509**, 21–32.
22. N.A. Lachant, W.B. Davidson and K.R. Tanika (1983) Impaired pentose phosphate shunt function in sickle cell disease: a potential mechanism for increased Heinz body formation and membrane lipid peroxidation. *American Journal of Haematology*, **15**, 1–13.
23. W. Wettersroem, G.J. Brewer, J.A. Warth, A. Mitchinson and K. Near (1984) Relationship of

glutathione levels and Heinz body formation to irreversibly sickled cell in sickle cell anaemia. *Journal of Laboratory and Clinical Medicine*, **103**, 589–596.
24. S.G. Sullivan and A. Stern (1984) Membrane protein changes induced by *t*-butyl hydroperoxide in red blood cells. *Biochimica Biophysica Acta*, **774**, 215–220.
25. A. Puppo, R. Cecchini, O.I. Aruoma, R. Bolli and B. Halliwell (1990) Scavenging of hypochlorous acid and of myoglobin-derived oxidants by the cardio-protective agent mercaptopropionyl glycine. *Free Radical Research Communications*, **10**, 371–381.
26. C. Rice-Evans, G. Okunade and R. Khan (1989) The suppression of iron release from activated myoglobin by physiological electron donors and desferrioxamine. *Free Radical Research Communications*, **7**, 45—54.

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