Red cell lipid peroxidation and antioxidant enzymes in iron deficiency

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Abstract: Whether iron deficient RBC in humans have a reduced, or an increased, susceptibility to lipid peroxidation was studied in the iron deficiency states of primary proliferative polycythaemia and iron deficiency anaemia and related to changes in the activities of iron-dependent and non-iron dependent antioxidant enzymes. Susceptibility of RBCs to lipid peroxidation was increased when expressed per g Hb. However, this was a result of the low RBC Hb giving an increased membrane lipid: Hb ratio in the incubations. Results were normal when expressed either per cell, or per ml, RBC. Glutathione reductase was normal. Increased RBC superoxide dismutase activity in iron deficiency may be explained by the younger RBC population and reductions in glutathione peroxidase and catalase activities by the microcytic hypochromic changes and the lack of availability of iron, respectively. There is no evidence of an increased susceptibility of RBC to lipid peroxidation in iron deficiency.

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Introduction

Iron-mediated oxidative damage has been demonstrated *in vivo* in normal red blood cells (RBC) (1). The constant auto-oxidation of haemoglobin generates superoxide radicals (O_2^-), which through spontaneous or enzymatic dismutation yield hydrogen peroxide. The involvement of free and haemoglobin-associated iron species in the generation of highly reactive radicals has recently been reviewed (2). Normal RBCs are resistant to oxidative damage through their anti-oxidant enzyme systems.

In iron overload, such as in β-thalassaemia, increased levels of RBC malonyldialdehyde (MDA), a secondary breakdown product of lipid peroxides, have been reported (3, 4). Increased lipid peroxidation after short-term iron loading *in vivo* in rats has also been documented (5). However, increased lipid peroxidation has also been reported in the converse situation of iron deficiency in rats (6). Based on the nature of iron toxicity (7), iron deficiency would not be expected to give increased lipid peroxidation, unless a decrease in the iron dependent enzyme catalase impairs the antioxidant mechanisms of RBC (8, 9, 10, 11). It is therefore unclear whether the ability of RBC to undergo lipid peroxidation is increased or decreased during human iron deficiency.

Patients with untreated iron deficiency anaemia (IDA), unrelated to other diseases, are uncommon. However RBC from patients with primary proliferative polycythaemia (PPP), treated by repeated venesection, show evidence of iron deficiency, with low MCV and MCH values, but normal Hb and reticulocyte numbers. Such RBC are otherwise unchanged in PPP with normal levels of glycolytic intermediates and enzyme levels of the Embden Meyerhof and the hexose monophosphate pathways (12, 13). Therefore PPP patients provide a human model of steady-state iron deficiency and thus an additional group of patients to those with IDA in which to study the effects of iron deficiency on RBC.

The present study examines the *in vitro* susceptibility to lipid peroxidation of human RBC in the iron deficiency states of PPP and iron deficiency anaemia (IDA) with respect to changes in the activities of the antioxidant enzymes, catalase, superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR). The results are presented in different units to take account of the changes in the physical constants of cell size, volume and Hb content that occur in iron deficiency.

Material and methods

Patients

14 patients with PPP, aged 56–74 years, 11 male: 3 female, diagnosed by standard criteria (14), and with indices of iron deficiency (Table 1) were studied. All had MCV values less than 80 fl. 5 patients, 1 male: 4 female, aged 28–42 yr, with iron deficiency anaemia (IDA) uncomplicated by a myeloproliferative disorder were also studied. Results were compared

Table 1. Haematological indices in iron-deficient primary proliferative polycythaemia (PPP), iron-deficiency anaemia (IDA) and normal control subjects

	Hb (g/dl)	PCV	MCV (fl)	MCH (pg)	Retics $\times 10^9$/l
Control subjects (14)	13.99 ±1.00	0.42 ±0.003	91.4 ±3.8	30.4 ±1.7	85 ±15
Treated PPP (14)	13.62 ±0.88	0.45 ±0.02	70.8 ±5.4	22.9 ±2.7	70 ±14
Iron-deficient patients (5)	8.54 ±2.35	0.29 ±0.06	64.6 ±13.0	19.7 ±4.8	62 ±8

Numbers of subjects/patients studied in parentheses. Means±SD.

with those of 14 healthy control subjects (7 male: 7 female, aged 28–55 yr).

Blood samples

Fresh blood (20 ml) was collected in lithium heparin. Routine blood counts were determined on EDTA-anticoagulated blood samples on a Coulter Model S-Plus IV counter (Coulter Electronics Ltd., Luton, England).

Spectrophotometric determinations were carried out on a LKB Ultrospec II spectrophotometer (LKB-Pharmacia Ltd., Milton Keynes, England).

Susceptibility to lipid peroxidation

This was measured using a modification of the method of Stocks & Dormandy (15) in which hydrogen peroxide is replaced by the more stable and commonly used lipid peroxide analogue, t-butyl hydroperoxide (t-BHP). Washed red cells (2.5% suspension) with a haemoglobin content of 7.5 mg/ml were added to an equal volume of 0.75 mmol/l tertiary butyl hydroperoxide (t-BHP) in phosphate buffered saline containing 1 mmol/l azide. At 0, 60, 75 and 90 min the product, malonyldialdehyde (MDA), was measured by its reactivity with thiobarbituric acid (TBA), the absorbance of which was measured at 535 nm (16). A calibration curve was constructed using malonyldialdehyde bis-diethyl acetal as a standard (range 0–2.5 μmol/l). Results were expressed as nmoles MDA produced per g Hb, 10^{10} RBC or ml RBC.

Enzyme studies

Heparinised red cells were washed three times with phosphate-buffered saline, pH 7.4. Packed RBCs were lysed with 9 vol 0.02 mol/l potassium phosphate buffer, pH 7.0, containing 0.5% w/v Triton X-100 (17) and sonicated on an MSC Soniprep 150 for 1 min. The haemolysate was stored in aliquots at −20°C. Reaction rates were measured at 37°C. Results were expressed as units per g Hb, 10^{10} RBC or ml RBC.

The activity of SOD was measured by the method of Flohe & Otting (18). The inhibition of cytochrome C reduction by SOD was plotted as a reciprocal absorbance change per min against the concentration of SOD standard. One unit of SOD was that giving 50% inhibition of cytochrome C reduction.

Glutathione peroxidase activity, comprising both the selenium dependent non-selenium dependent peroxidase activity was measured by the method of Paglia & Valentine (19) with 1.5 mmol/l cumene hydroperoxide as substrate to measure total peroxidase activity (20). RBC lysates were diluted 1:1 in double strength Drabkin's reagent to convert Hb to cyanomet Hb prior to assay. One unit of enzyme activity was defined as 1 μmol NADPH oxidised per min.

Glutathione reductase was determined as described by Xia et al. (21). One unit of enzyme activity was equal to 1 μmol NADPH oxidised per min.

Catalase was measured as described by Beutler (22), using hydrogen peroxidase as substrate. One unit of enzyme activity was equivalent to 1 μmol substrate converted per min.

Results were analysed by Student's t-test between control *vs* PPP or IDA.

Results

The haematological indices of the control subjects and PPP and IDA patients are shown in Table 1. The serum iron and iron-binding capacity values were 2.8–12.4 μmol/l and 63–91 μmol/l respectively for PPP, and 3.4–6.9 μmol/l and 80–94 μmol/l respectively for IDA (Normal ranges: serum iron 13–32 μmol/l, TIBC 45–70 μmol/l). To avoid complicating effects of hypochromia and microcytosis, all data have been expressed as units per g Hb, 10^{10} RBC and ml RBC.

Susceptibility to lipid peroxidation

Mean MDA production after stressing RBCs with t-BHP at 0 and 90 min is shown in Table 2. Mean MDA production of the t-BHP stressed RBCs of PPP was not significantly different from the mean control value, irrespective of the units used. In IDA, only mean RBC MDA generation expressed in relation to g Hb was significantly increased ($p < 0.01$).

Superoxide dismutase

In PPP, mean SOD activity was significantly increased per g Hb ($p < 0.005$) and per ml RBC ($p < 0.05$), but decreased per 10^{10} RBC ($p < 0.05$) (Table 3), all compared with normal controls.

Table 2. Tertiary butylhydroperoxide (t-BHP) stressed RBC malonyldialdehyde production in primary proliferative polycythaemia (PPP), iron-deficiency anaemia (IDA) and in control subjects

	Controls (14)		PPP (14)		IDA (5)	
Time (min)	0	90	0	90	0	90
U/g Hb	0.03±0.01	0.25±0.05	0.04±0.02 (NS)	0.32±0.08 (NS)	0.07±0.05 (NS)	0.39±0.05 (p<0.01)
U/10^{10} RBC	0.11±0.09	0.78±0.12	0.09±0.05 (NS)	0.72±0.16 (NS)	0.12±0.06 (NS)	0.79±0.24 (NS)
U/ml RBC	11.7±5.3	89.8±17.4	11.8±6.0 (NS)	101.8±25.0 (NS)	21.0±13.5 (NS)	126.0±21.2 (NS)

Numbers of patients/subjects studied in parentheses.
Means±SD.
NS=not significant.

Table 3. Red cell superoxide dismutase and glutathione reductase activities in iron-deficient primary proliferative polycythaemia (PPP), iron-deficiency anaemia (IDA) and in control subjects

	Superoxide dismutase			Glutathione reductase		
Units	U/g Hb	U/10^{10} RBC	U/ml RBC	U/g Hb RBC	U/10^{10} RBC	U/ml
Controls (14)	1571±186	481±60	522±66	4.6±1.6	1.4±0.5	1.5±0.6
PPP (14)	1858±261 (p<0.005)	425±60 (p<0.05)	609±119 (p<0.05)	5.1±1.4 (NS)	1.5±0.3 (NS)	1.6±0.4 (NS)
IDA (5)	2275±440 (p<0.001)	446±103 (NS)	662±114 (p<0.01)	5.4±2.3 (NS)	1.0±0.3 (NS)	1.6±0.6 (NS)

Numbers of patients/subjects studied in parentheses.
Means±SD.
NS=not significant.

In IDA, mean RBC SOD activity was also increased per g Hb ($p<0.001$) and per ml RBC ($p<0.01$), but mean SOD activities per 10^{10} RBC in the IDA group and the control group were not statistically different.

Glutathione reductase

The mean activity of the enzyme expressed in all three ways was normal in both PPP and IDA (Table 3).

Glutathione peroxidase

Mean GPx activity per 10^{10} RBC was significantly decreased in PPP ($p<0.001$) and IDA ($p<0.01$), but normal when expressed in other units (Table 4).

Catalase

The mean catalase activity was normal in PPP (Table 4), but there were significant correlations between catalase expressed per g Hb, 10^{10} RBC or ml RBC and MCV ($p<0.01$; $r=0.69, 0.76, 0.73$ respectively).

In IDA, the mean catalase activity was significantly decreased per 10^{10} RBC ($p<0.001$) and per ml RBC ($p<0.01$), but normal per g Hb (Table 4).

Discussion

The problem of expressing results in iron deficiency has been clearly recognized (10, 11). Earlier, Hjelm (23) concluded that no expression was satisfactory for all circumstances. However, the most physiological and the least ambiguous is the activity per ml. The activity per cell may change because smaller cells will contain less enzyme, whereas the activity per gram Hb would increase if the MCHC falls, as it does in iron-deficient RBCs.

Thus RBC lipid peroxidation was normal in iron deficiency when MDA generation was expressed per cell or per ml. There was an increased production of MDA per gHb in both the patient groups studied but this was only significant in IDA, probably because the iron-deficient changes were more marked in this group. This apparent increase is explained by the decrease in Hb in each cell giving an increase in the number of red cells, and hence increased oxidisable

Table 4. Red-cell glutathione peroxidase and catalase activities in primary proliferative polycythaemia (PPP), iron deficiency anaemia (IDA) and in control subjects

	Glutathione peroxidase			Catalase		
	U/g Hb	U/10^{10}	U/ml	U/g Hb	U/10^{10}	U/ml
Controls (14)	26.1±4.7	7.98±1.44	8.67±1.51	20.9±2.9	6.29±0.88	6.91±0.93
PPP (14)	23.8±7.1 (NS)	5.36±1.20 ($p<0.001$)	7.38±2.01 (NS)	23.5±5.6 (NS)	5.29±1.43 (NS)	7.18±1.64 (NS)
IDA (5)	28.1±8.4 (NS)	5.44±1.60 ($p<0.01$)	8.18±2.40 (NS)	18.2±2.4 (NS)	3.56±0.87 ($p<0.001$)	5.14±0.60 ($p<0.01$)

Numbers of subjects/patients studied in parentheses.
Means±SD.
NS=not significant.

membrane lipid, required to standardize the incubation with respect to a constant amount of haemoglobin.

In PPP the decreased SOD activity per 10^{10} RBC and increased activity per g Hb reflect the decreased cell volume and reduced MCHC respectively. However, the SOD activity per ml RBC is increased in both the iron deficient PPP and IDA groups. This probably reflects a true increase in RBC SOD in iron deficiency. Creatine is a more sensitive index of the age of the RBC (24) and is increased in iron deficiency even when the reticulocyte count is normal (25). Thus, although the reticulocyte numbers were normal the increased SOD may still be due to a younger RBC population in iron deficiency.

Catalase is an iron-dependent enzyme and would be expected to be decreased in iron deficiency. The lack of any change in catalase activity in PPP was probably because the iron deficiency was less severe than in the IDA group. The significant correlation between catalase expressed in all three ways and MCV in PPP suggests a decline in catalase with increasing severity of iron deficiency. Furthermore, previous studies have also reported decreased catalase per 10^{10} RBC and per ml RBC in IDA (8, 9, 10, 11).

However, lipid peroxidation per cell was normal in IDA and the physiological role of catalase in RBC is uncertain. Earlier studies emphasised its relatively low affinity for H_2O_2 under physiological conditions (26, 27), but more recently both catalase and GPx were found to be equally active in the detoxification of H_2O_2 (28). Moreover, with the complete catalase deficiency described in Japanese (29) and Swiss families (30), no haemolysis occurs. Thus, decreased catalase activity in iron deficiency is probably not detrimental to the stability of the RBC.

Glutathione peroxidase activity in IDA and PPP was only significantly decreased when expressed per cell. This is probably due to the reduced cell size. Decreased GPx per cell has been reported in IDA (11, 31, 32, 33), and it has been suggested that this may cause decreased red cell survival (34, 35, 36). However, it is probable that, as in the present study, a decrease in GPx activity is simply due to the choice of the denominator in the expression of the results and is not a true decrease. Results being normal when expressed per ml and g Hb. Furthermore, the ability to maintain a high ratio of reduced to oxidised glutathione is unaltered in these states as RBC GR activity was unchanged in both IDA and PPP.

In conclusion, RBC lipid peroxidation is not increased in human iron deficiency. In addition, contrary to a study in myeloproliferative disorders that included PPP (37), susceptibility of RBC to lipid peroxidation is not increased in patients with PPP. Glutathione reductase activity is normal in IDA, unlike reported values of decreased GR per cell in iron-deficient rabbits (33). Significant changes in GPx and catalase activities can be explained by the mode of expressing the results and the decrease of iron respectively, and do not signify any intrinsic functional abnormality in RBC antioxidant defences in iron deficiency.

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