Ascorbate removes key precursors to oxidative damage by cell-free haemoglobin *in vitro* and *in vivo*

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Haemoglobin initiates free radical chemistry. In particular, the interactions of peroxides with the ferric (met) species of haemoglobin generate two strong oxidants: ferryl iron and a protein-bound free radical. We have studied the endogenous defences to this reactive chemistry in a rabbit model following 20 % exchange transfusion with cell-free haemoglobin stabilized in tetrameric form [via cross-linking with bis-(3,5-dibromosalicyl)fumarate]. The transfusate contained 95 % oxyhaemoglobin, 5 % methaemoglobin and 25 μM free iron. EPR spectroscopy revealed that the free iron in the transfusate was rendered redox inactive by rapid binding to transferrin. Methaemoglobin was reduced to oxyhaemoglobin by a slower process ($t_{1/2} = 1$ h). No globin-bound free radicals were detected in the plasma. These redox defences could be fully attributed to a novel multifunctional role of plasma ascorbate in removing key precursors of oxidative damage. Ascorbate is able to effectively reduce plasma methaemoglobin, ferryl haemoglobin and globin radicals. The ascorbyl free radicals formed are efficiently re-reduced by the erythrocyte membrane-bound reductase (which itself uses intra-erythrocyte ascorbate as an electron donor). As well as relating to the toxicity of haemoglobin-based oxygen carriers, these findings have implications for situations where haem proteins exist outside the protective cell environment, e.g. haemolytic anaemias, subarachnoid haemorrhage, rhabdomyolysis.

Key words: ascorbate, blood substitute, ferryl, free radical, haemoglobin, oxidative stress.

INTRODUCTION

Haemoglobins and myoglobins are involved in oxygen transport and storage. As a consequence of this function and because of the redox properties of the iron, these proteins also generate reactive oxygen species [1]. Autoxidation of the oxy derivative (Fe^{2+}) leads to non-functional ferric haem (Fe^{3+}) and superoxide ion ($O_2^{\bullet -}$), which subsequently dismutates to generate H_2O_2. These species can ultimately damage the protein and/or the haem group [2]. An essential intermediate in the pathway leading to this damage is the ferryl haem (Fe^{4+}), itself formed through the reaction of the ferric haem with H_2O_2 [3]. Accompanying the formation of the ferryl haem is a protein/porphyrin-based radical cation ($P^{+\bullet}$) (see eqn 1) [4,5].

$$P\text{-}Fe^{3+} + H_2O_2 \rightarrow P^{+\bullet}Fe^{4+}{=}O^{2-} + H_2O \quad (1)$$

Ferryl haem and the radical can also be extremely toxic, notwithstanding their transient existence. These oxidative cascades can be damaging because: (i) peroxide is a powerful oxidant known to produce cellular damage [6], (ii) both the ferryl haem and protein-based radicals can initiate oxidation of lipids, nucleic and amino acids by abstraction of hydrogen atoms [7,8], and (iii) haem modification can lead to highly toxic haem to protein-cross-linked species and to the loss of haem and the release of the 'free' iron [2,9–11].

The potential for haemoglobin-mediated peroxidative damage exists whenever the protein is removed from the protective environment of the erythrocyte. This would occur, for example, during spontaneous erythrocyte haemolysis or in haemolytic anaemias (e.g. sickle-cell anaemia [12]). We have shown that myoglobin induces kidney damage following crush injury (rhabdomyolysis) by exactly this peroxidative mechanism, rather than by free-iron-catalysed Fenton chemistry as was thought previously [13,14]. In contrast, there has been very little research carried out in humans or other animals on haemoglobin oxidative kinetics and possible contributions of these reactions to the overall toxicity of free haemoglobin. However, we have shown recently that haemoglobin can cause similar damage *in vivo* when it is released from the erythrocyte in subarachnoid haemorrhage [15]. Furthermore, uncontrolled haem-mediated oxidative reactions of cell-free haemoglobin (developed as a blood substitute) have emerged as an important potential pathway of toxicity, either directly or via interactions with cell signalling pathways [16]. The toxicity of ferryl haemoglobin has been demonstrated in an endothelial cell culture model system of ischaemia/reperfusion [17] and in cells that lack their antioxidant mechanisms such as glutathione [18]. Ferryl haemoglobin can cause cell injury, including apoptosis and necrotic cell death. Perfusion of rat intestine with chemically modified haemoglobin has been shown to cause localized oxidative stress, leading to leakage of the mesentery of radiolabelled albumin [19]. Importantly, the cyanomet derivative of this haemoglobin in which the haem iron is blocked with cyanide and is unavailable to enter a redox reaction produced no cellular changes.

In spite of the presence of a number of well-known antioxidant defence mechanisms, very little is known about the precise mechanism(s) by which haemoglobin oxidative toxicity is controlled *in vivo*. *In vitro*, the properties of ascorbate (vitamin C)

Abbreviations used: DBBF, bis-(3,5-dibromosalicyl)fumarate; MAP, mean arterial pressure.

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as a reductant are well established in a range of biological systems [20]. Reperfusion of isolated ischaemic rat hearts with ascorbate decreased myocardial injury [20,21]; other investigations have shown that ascorbate directly suppressed ferryl myoglobin in isolated hearts [22] and improved cardiac function in a model of magnesium deficiency [23]. Consistent with these observations, recent experiments have demonstrated a direct correlation between the ascorbate-mediated reduction of ferryl myoglobin and haemoglobin and inhibition of apoptosis in cultures of endothelial cells exposed to peroxide [24].

The infusion of cell-free haemoglobin as blood substitutes in large quantities provides an opportunity to explore the interplay between haemoglobin oxidative side reactions and plasma antioxidant mechanisms. In this paper, we report for the first time the results of studies designed to monitor the oxidative consequences of administering cell-free haemoglobin into an animal model of partial exchange transfusion and reveal a novel multifunctional role of ascorbate in preventing haem toxicity *in vivo*.

EXPERIMENTAL

Haemoglobin solutions

For transfusion studies, an $8.2\ g \cdot dl^{-1}$ solution of haemoglobin cross-linked between the α-subunits with bis-(3,5-dibromosalicyl)fumarate in Ringers lactate (Hb–DBBF; Walter Reed Army Institute of Research, Washington, DC, U.S.A.) was used. For the *in vitro* studies, both Hb–DBBF and 'control' HbA_o were used. The latter was prepared from human volunteer blood using the method of Antonini and Brunori [25] with the addition of ion-exchange chromatography on DEAE–Sephadex A50 [26] to remove contaminating catalase. HbA_o was stored in the CO-bound form at 77 K to prevent autoxidation. Methaemoglobin was prepared from carboxyhaemoglobin as described previously [27].

Preparation of erythrocytes and erythrocyte ghosts

Whole blood was collected in EDTA from healthy volunteers under informed consent and with approval of the National Institutes of Health, Institution Review Board (protocol # 03120B). Whole blood in EDTA was washed with PBS buffer, pH 7.4, centrifuged at 1000 ***g*** for 10 min, followed by removal of non-erythrocyte components. The process was repeated five times to isolate packed erythrocytes. Ghost erythrocytes (unsealed) were prepared according to the methodology described by Steck and Kant [28].

Reduction of metHb–DBBF by erythrocytes and erythrocyte ghosts

Aliquots of erythrocytes or ghost erythrocytes were prepared in 1.5 ml conical tubes ($n = 3$ tubes per time point) and diluted appropriately to simulate 0, 20, 30 and 40 % haematocrit levels using PBS buffer, pH 7.4, supplemented with 0.1 % (w/v) human serum albumin (ZBL) and 0.1 % (w/v) glucose (Sigma). Then, 100 μM metHb–DBBF and 100 μM ascorbate were added. Immediately thereafter, all tubes were placed in a circulating water bath at 37 °C with gentle agitation. Samples were evaluated at baseline and every 30 min for a maximum of 2 h. Briefly, samples were centrifuged at 4000 ***g*** for 10 min to separate the erythrocytes from metHb–DBBF. The supernatant was diluted and measured in a photodiode array spectrophotometer (Hewlett Packard 8453) scanning from 450 to 700 nm with formation of oxyHb–DBBF and reduction of metHb–DBBF monitored at 576 and 630 nm respectively. MALDI (matrix-assisted laser-desorption ionization)-MS of the metHb–DBBF samples after centrifugation could not detect any contamination from HbA_o, indicating that haemolysis was insignificant in the course of the study.

Isovolaemic haemodilution in anaesthetized rabbits

The animal protocol was approved by the French National Ethics Committee (licence no. 006101), and the experiments were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 80-23, revised 1985). Male New Zealand white rabbits (La Garenne, Villey Saint-Etienne, France), weighing 2.7 ± 0.3 kg, were acclimated for 1 week after arrival with free access to food and water. At the end of the experiments, the animals were killed with an excess dose of pentobarbital. At 3 h before the study the animals were anaesthetized with halothane (Blamont, Neuilly-sur-Seine, France) and chronically implanted with two venous and two arterial heparin-filled polyethylene catheters. The conscious animals were then subjected to exchange transfusion (20 % of total blood volume) with saline ($n = 7$) or Hb–DBBF solution ($n = 6$). MAP (mean arterial pressure), heart rate, blood pH, haemoglobin concentration and haematocrit were monitored before exchange transfusion and for a 3 h period of isovolaemic haemodilution. The duration of the exchange transfusion was approx. 30 min; the initial time points when samples were taken post-transfusion ($t = 5$) refers to the time after the end of this period (and therefore some *in vivo* changes will have occurred owing to DBBF injected at the beginning of the transfusion period).

EPR

EPR spectral measurements were made on blood samples which were collected in sterile heparinized syringes. Whole blood and plasma samples were transferred immediately into EPR tubes, frozen in methanol, cooled in solid CO_2 and placed in liquid N_2 storage until analysis by EPR spectroscopy. All low-temperature EPR spectra were measured at 10 K in a Bruker EMX spectrometer, with a spherical high-quality Bruker resonator SP9703 and an Oxford Instruments liquid helium system. In all cases, samples of water were run under the same conditions as the samples and readings were subtracted to remove background signals from the cavity, before subsequent analysis. Quantification of EPR signals in frozen blood was as described previously [29], using pure metHb, ferric transferrin and Cu(II)-EDTA and as standards for $g = 6$, $g = 4.3$ and $g = 2$ signals respectively. In pure haemoglobin solutions, separate quantification of the haemoglobin tryptophan peroxyl radical from the haemoglobin tyrosyl radical was performed by the technique of subtraction with a variable coefficient described previously [30], focusing initially on the $g = 2.033$ region where only the peroxyl radical contributes. The separate quantification of radical species was not possible in whole blood because of the low radical concentrations and the presence of ceruloplasmin. Room-temperature EPR measurements were performed using a Wilmad WG-813-TMS aqueous cell.

The concentration of labile iron able to bind to transferrin directly in the Hb–DBBF solution was determined *ex vivo*. The iron bound to transferrin in venous plasma samples from one of the control experiments in rabbits was measured by EPR spectroscopy. These samples were then allowed to thaw. A solution of the Hb–DBBF was added to give a dilution of 20 %, as in the *in vivo* experiment. The solutions were left for 1 h at room temperature (295 K) before being refrozen, and the increase in the amount of iron bound to transferrin was measured by EPR.

HPLC analysis of peroxide-modified haemoglobin

metHb–DBBF was reacted with H_2O_2 and analysed for the loss of the haem and the formation of cross-links between the haem group and the globin molecule (termed Hb-H) by reverse-phase

HPLC using a method adapted from that of Osawa and Korzekwa [31] and described in detail in [10].

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 3.0 for Windows (GraphPad Software, San Diego, CA, U.S.A.). The immediate effect of the transfusion was tested using Student's paired t test, before and after transfusion. Subsequent changes after transfusions with time were tested using a one-factor ANOVA for repeated measures, testing for a significant linear trend. Comparisons between control and Hb–DBBF-transfused groups were made using two-way ANOVA for repeated measures. Time points where there was a significant difference were determined using a Bonferroni post test. $P < 0.05$ was considered significant in all cases. Statistical comparisons of ascorbate reduction of metHb–DBBF by erythrocytes and erythrocyte ghosts was performed by a one-way ANOVA followed by the a priori test to determine the level of significance between means. Comparisons of the radical steady states in isolated HbA_o and Hb–DBBF following peroxide treatment were compared using Student's unpaired t tests.

RESULTS

The effect of peroxides on haemoglobin can be assayed by optical and magnetic techniques. Figure 1(a) shows the effect of adding a 2 molar excess of H_2O_2 (2 mol of H_2O_2 per mol of haem) on the optical spectrum of ferric (met) HbA_o. Peaks appeared at 545 and 585 nm, characteristic of oxidation of the ferric (Fe^{3+}) to the ferryl ($Fe^{4+}=O^{2-}$) redox state [32]. Production of the ferryl species was confirmed by the addition of sulfide, which generates sulfhaemoglobin from ferryl, indicated by the peak at 620 nm [22]. The addition of H_2O_2 to Hb–DBBF generates an identical spectrum with that of ferrylHbA_o (Figure 1b). The stoichiometry of ferryl formation is also identical in the two haemoglobins (Figure 1c) with one molecule of H_2O_2 converting one molecule of met (Fe^{3+}) into ferryl ($Fe^{4+}=O^{2-}$). Therefore although H_2O_2 is a two-electron oxidant, only one electron is used to oxidize the haem. The other electron oxidizes the protein, generating a protein radical [27]. This radical can be detected magnetically, by the use of EPR spectroscopy.

Methaemoglobin has a high-spin ferric haem, with characteristic EPR spectral features at $g = 6$ and $g = 1.99$ (Figure 1d). Following the addition of H_2O_2, the haem is oxidized to ferryl (which is low-spin and EPR-undetectable). Hence there is a decrease in the $g = 6$ signal. Accompanying this decrease, there is a formation of free radicals on the globin, indicated by the sharp signal at $g = 1.99$. This spectrum is, in fact, composed of a complicated mixture of radical species situated on tyrosine and tryptophan residues [27,33–35]. An expansion of the $g = 2$ region shows that the radical species formed in HbA_o are all also formed in Hb–DBBF (Figure 1e). Hb–DBBF therefore is capable of forming both ferryl iron and free radicals *in vitro*. The overall kinetic profile of the formation and decay of the haemoglobin radicals is not fundamentally altered compared to HbA_0 [36]. However, at 30 s post-peroxide addition, the concentrations of both the tyrosine radical (+40 %) and the tryptophan peroxyl radical (+70 %) are significantly increased in the case of Hb–DBBF when compared with HbA_o, consistent with previous measures of a slightly enhanced rate of reactivity of peroxide with Hb–DBBF [37].

To explore the likelihood of these species being formed *in vivo*, exchange transfusions were performed in a conscious rabbit model ($n = 6$). Comparisons were made with controls of an approx. 20 % (blood volume) exchange-transfused with Hb–DBBF re-infused with autologous blood (Table 1). The measured haematocrit fell by 23 % in the Hb–DBBF animals, but was unchanged in the controls. The basic physiological results were consistent with previous studies [38,39]. Immediately following transfusion, there was a significant drop in the heart rate in the Hb–DBBF animals and a rise in blood pressure. These effects were not seen in the control animals and are consistent with the known hypertensive effects of Hb–DBBF, believed to be due to its direct reactions with nitric oxide [40]. Blood pH did not change significantly in either control or Hb–DBBF-treated animals. Total haemoglobin fell slightly in Hb–DBBF-treated animals (immediately following the transfusion), owing to the concentration of haemoglobin in the transfused Hb–DBBF solution (8.2 g/dl) being lower than the average concentration in the blood (14.0 g/dl). All of the significant changes in the haematocrit, haemoglobin concentrations, heart rate and blood pressure that occurred with Hb–DBBF occurred during the time of the transfusion. No changes were seen in these parameters over the remaining 3 h of the study.

EPR measurements on whole blood can reveal information about a range of paramagnetic species, such as free radicals and transition metals. All the EPR data reported here are illustrated by spectra from individual animals, but identical changes were seen in all animals (Table 1). Figure 2(a) shows the five compounds readily identifiable by EPR in whole blood. They correspond to iron in methaemoglobin, catalase and transferrin, copper in ceruloplasmin and the globin free radical(s). Comparisons with plasma and erythrocyte spectra confirm that the catalase, methaemoglobin and free radical signals are only seen in erythrocytes, and the transferrin and ceruloplasmin signals are only seen in plasma. We have previously identified the free radical signal as being primarily attributable to the tyrosine radical associated with the addition of H_2O_2 to haemoglobin [41], i.e. there is direct *in vivo* evidence that within the erythrocyte the reactions outlined in Figure 1 result in a detectable steady-state level of globin-bound free radicals even in control animals or healthy human volunteers [42].

A comparison of the spectrum of whole blood before and after the exchange transfusion with Hb–DBBF shows a large increase in the methaemoglobin signal at $g = 6$ (Figures 2b and 2c). In the plasma pre-transfusion there is, as expected, no $g = 6$ methaemoglobin EPR signal (Figure 2d). Following transfusion, a similar increase is seen here, as was observed for the whole blood. Although most (> 90 %) of the Hb–DBBF (pre-transfusion) is present in the oxyhaemoglobin form, 5.1 ± 1.2 % is present as methaemoglobin. This corresponds to approx. 220 μM methaemoglobin in the transfused solution. Therefore a 20 % exchange transfusion would be expected to generate approx. 40–50 μM methaemoglobin. These levels are equivalent to those observed in the plasma (Table 1). Thus the data are consistent with the simple explanation that the methaemoglobin in the transfused Hb–DBBF solution appears in the plasma, essentially unchanged, at least initially. (Note that after the transfusion is over, methaemoglobin decreases. The discrepancy between the 40–50 μM methaemoglobin in the injected solution and the 33 μM that we observe at $t = 0$ can be explained by the reduction of methaemoglobin injected early in the 30 min transfusion process.)

The only other significant change (Table 1) in the EPR spectra immediately after transfusion is an increase in the iron loading of plasma transferrin (resulting in an increase in the $g = 4.3$ signal). The data could be consistent with the transfusion resulting in a release of iron from erythrocytes or damage to the Hb–DBBF. However, following the initial increases, the $g = 4.3$ transferrin signal was unchanged with time (Table 1), suggesting that the

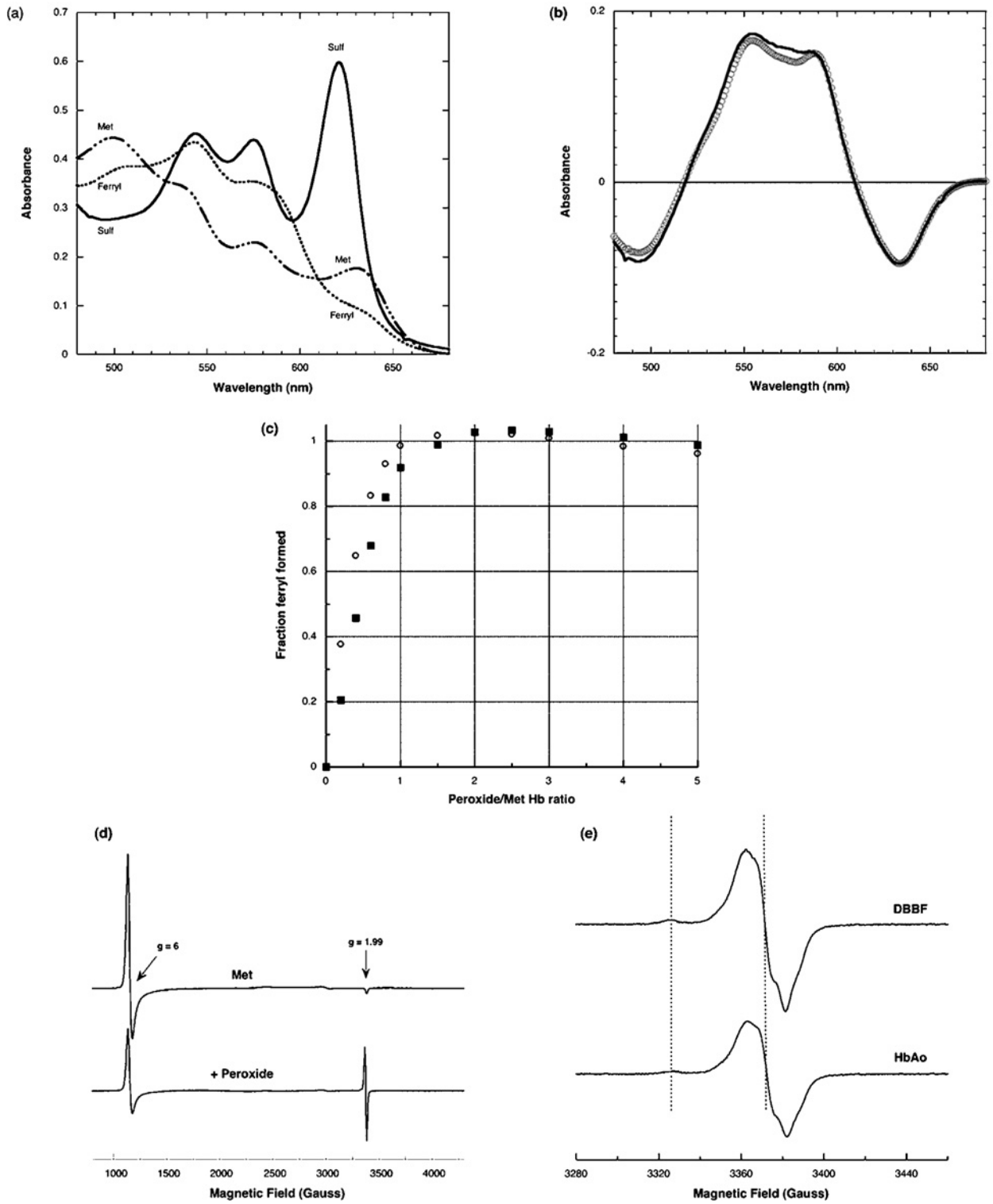

Figure 1 Optical and EPR spectra following peroxide addition to methaemoglobins

(**a**) Optical spectra of metHbA_0 (50 μM) before (Met) and 2 min after (Ferryl) the addition of 100 μM H_2O_2, with subsequent addition of 2 mM sulfide (Sulf). (**b**) Difference spectrum (ferryl minus met) for HbA_0 (−) and Hb–DBBF (○). (**c**) Titration of ferryl formation (determined from absorbance change at 545 minus 700 nm) against peroxide concentration added, assuming maximum formation at saturating peroxide concentrations (○, Hb–DBBF; ■, HbA_0). Experimental conditions: sodium phosphate (50 mM) containing DTPA (diethylenetriaminepenta-acetic acid) (20 μM), pH 7.4, at 37 °C. (**d**) EPR spectra of metHbA_0 (50 μM), before and 30 s after the addition of H_2O_2 (100 μM), in sodium phosphate (50 mM) containing DTPA (50 μM), pH 7.4, at 37 °C. EPR conditions: temperature, 10 K; microwave power, 3.2 mW; modulation amplitude, 4 G; receiver gain, 1×10^4; microwave frequency, 9.47 GHz; time constant, 81.92 ms; scan rate, 23 G/s. (**e**) Close up of free radical region of EPR spectra following peroxide addition to metHbA_0 and metHb–DBBF under experimental conditions identical with those in (**d**). Illustrated features relate to tyrosine ($g = 2.005$) and tryptophan peroxyl radicals ($g = 2.033$). EPR conditions: temperature, 10 K; microwave power, 12.69 μW; modulation amplitude, 3 G; microwave frequency, 9.47 GHz; receiver gain, 3.99×10^4; time constant, 81.92 ms; number of scans, 15; scan rate, 2.9 G/s. Total radical concentrations (means $\pm$ S.D.; $n = 6$): HbA_0 tyrosine, 1.31 ± 0.09 μM; Hb–DBBF tyrosine, 1.88 ± 0.14 μM; HbA_0 tryptophan peroxyl, 0.26 ± 0.02 μM; Hb–DBBF tryptophan peroxyl, 0.44 ± 0.03 μM. ($P < 0.001$ for the increase seen in both radicals in the case of Hb–DBBF.)

Table 1 Physiological and EPR parameters following 20% transfusion with Hb–DBBF

Physiological and EPR data for animals undergoing control transfusions of autologous blood ($n=7$) and those undergoing transfusions with DBBF ($n=6$). All data are means ± S.D. In the Hb–DBBF group, data were corrected for the blood removed by the drop in haematocrit. The methaemoglobin concentration in the EPR samples was calculated by comparing the size of the strong high-field feature ($g=6$) with that of a pure methaemoglobin standard of known concentration. To remove the problem of the low-field ($g=1.99$) feature of the methaemoglobin signal interfering with the quantification of the other signals, the blood/plasma EPR spectra had the appropriate level of a pure haemoglobin spectrum subtracted from them before quantification of the other signals in this region. The ceruloplasmin and erythrocyte globin radical concentrations were then calculated by comparison of the second integrals of the respective signals with a copper EDTA standard, measured at non-saturating conditions for all species. The concentration of iron bound to transferrin was calculated by comparison of transferrin signals with that of a standard of human ferric transferrin of known concentration. Transferrin standards were prepared by the addition of atomic absorption standard iron in 1% HCl to excess human apotransferrin (a gift from Dr Robert Evans, King's College London, London, U.K.). Two-way ANOVA showed significant differences between control and Hb-DBBF for the following parameters: MAP, haematocrit, transferrin-bound iron and methaemoglobin. bpm, beats per minute.

		Time (min after transfusion)						
Parameter		Pre- transfusion control	5	15	30	60	120	180
MAP (mm Hg)	+DBBF	80.8 ± 11.9	93.8 ± 9.2*	91.7 ± 10.7	98.0 ± 13.0†	94.4 ± 9.1†	88.1 ± 12.7	92.3 ± 7.7†
	Control	80.1 ± 12.9	81.1 ± 11.5	75.5 ± 13.5	78.3 ± 12.8	73.3 ± 8.8	71.9 ± 10.5	70.4 ± 9.9
Heart rate (bpm)	+DBBF	264.2 ± 21.5	234.2 ± 30.4*	219.2 ± 41.5	232.5 ± 32.0	233.3 ± 22.3	226.0 ± 35.8	240.0 ± 34.5
	Control	249.3 ± 25.2	246.7 ± 25.2	248.3 ± 27.3	245.0 ± 32.7	237.5 ± 25.6	241.4 ± 26.1	229.3 ± 28.2
Blood pH	+DBBF	7.38 ± 0.04	7.40 ± 0.03	7.40 ± 0.04	7.40 ± 0.04	7.41 ± 0.05	7.42 ± 0.03	7.43 ± 0.04
	Control	7.37 ± 0.06	7.39 ± 0.03	7.39 ± 0.02	7.39 ± 0.03	7.39 ± 0.03	7.38 ± 0.02	7.40 ± 0.03
Haematocrit	+DBBF	40.9 ± 3.6	31.5 ± 3.6*	30.9 ± 3.6†	30.5 ± 3.2†	31.0 ± 3.5†	29.8 ± 3.0†	29.7 ± 3.0†
	Control	37.6 ± 3.0	36.6 ± 2.8	37.0 ± 2.3	36.7 ± 2.2	37.0 ± 1.9	35.0 ± 2.2	35.0 ± 2.4
Total Hb (g/dl)	+DBBF	12.5 ± 1.7	11.1 ± 1.0*	10.9 ± 1.2	11.0 ± 1.2	11.1 ± 1.3	10.7 ± 1.0	10.5 ± 0.5
	Control	11.9 ± 0.9	11.4 ± 0.6	11.7 ± 0.5	11.7 ± 1.3	11.4 ± 0.7	11.2 ± 1.1	10.8 ± 0.7
Ceruloplasmin (μM)	+DBBF	1.53 ± 1.27	1.48 ± 0.91	1.60 ± 1.32	1.73 ± 1.45	1.87 ± 1.57	1.71 ± 1.75	1.62 ± 1.60
	Control	2.47 ± 1.81	2.29 ± 1.71	2.37 ± 1.84	2.35 ± 1.81	2.37 ± 1.71	2.35 ± 1.81	2.41 ± 1.75
Transferrin iron (μM)	+DBBF	11.40 ± 2.03	17.14 ± 3.55*	17.50 ± 3.43†	18.16 ± 3.69†	18.68 ± 3.56†	20.03 ± 4.54†	18.79 ± 5.66†
	Control	10.74 ± 4.09	9.55 ± 3.01*	9.47 ± 3.52	9.46 ± 3.59	9.23 ± 3.26	8.56 ± 2.71	8.08 ± 2.30
Erythrocyte radical (μM)	+DBBF	0.083 ± 0.061	0.064 ± 0.026	0.071 ± 0.033	0.089 ± 0.031	0.047 ± 0.026	0.058 ± 0.062	0.051 ± 0.046
	Control	0.053 ± 0.012	0.049 ± 0.017	0.041 ± 0.007	0.088 ± 0.075	0.046 ± 0.015	0.046 ± 0.011	0.045 ± 0.018
MetHb (μM)	+DBBF	3.38 ± 1.23	33.36 ± 9.75*‡	29.09 ± 8.08†	23.17 ± 8.11†	15.17 ± 7.00†	8.21 ± 3.02	6.48 ± 2.95
	Control	2.69 ± 1.05	2.49 ± 0.82	2.56 ± 0.80	2.63 ± 0.90	2.41 ± 0.71	2.64 ± 0.55	2.61 ± 0.81

* Immediate post transfusion value ($t=5$) significantly different from pre-transfusion value.
† Significantly different from control (two-way ANOVA with Bonferroni post test).
‡ Significant linear trend (one-way ANOVA) from post transfusion ($t=5$) to the end of the study ($t=180$).

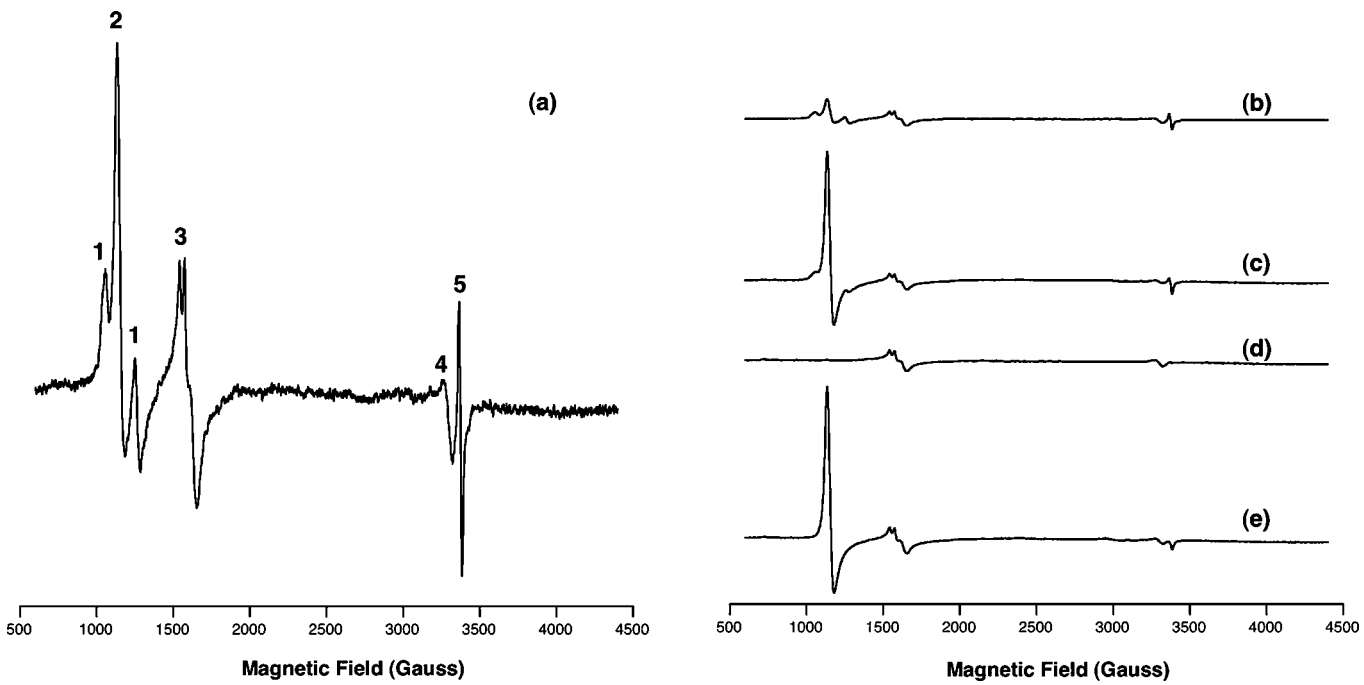

Figure 2 EPR spectra of blood and plasma following Hb–DBBF transfusion

(**a**) EPR spectrum of control rabbit venous blood measured at 10 K illustrating the following spectral features: 1. high-spin ferric haem iron in catalase, 2. high-spin ferric haem iron in methaemoglobin, 3. high-spin ferric non-haem iron in transferrin, 4. cupric copper in ceruloplasmin, 5. globin free radical. Experimental conditions: temperature, 10 K; microwave power, 3.18 mW; microwave frequency, 9.47 GHz; modulation frequency, 100 kHz; modulation amplitude, 5 G; number of scans, 1; gain, 2×10^4; time constant, 81.92 ms; scan rate 23 G/s. (**b**) Venous blood before transfusion. (**c**) Venous blood after transfusion. (**d**) Venous plasma before transfusion. (**e**) Venous plasma after transfusion.

transfusate itself was the source of this iron. The Hb–DBBF solution contains only very small amounts of EPR-detectable non-haem iron; this could not account for the signal directly ($< 0.05\ \mu$M, results not shown). However, the data are consistent with the presence of EPR-undetectable low-molecular-mass iron loosely bound to the protein solution. Upon transfusion, this iron would be rapidly scavenged by apotransferrin, producing the increase in the transferrin EPR signal. We tested this by adding the Hb–DBBF solution to rabbit plasma outside the animal (at the same 20 % dilution as if it were being added *in vivo*). There was a significant increase in the iron concentration bound to transferrin (from $18.5 \pm 1.0\ \mu$M to $23.5 \pm 2.0\ \mu$M; $n = 6$; $P < 0.001$). The mean difference in ferric transferrin observed in this *in vitro* experiment (5.0 ± 0.5) is consistent with that seen *in vivo* ($5.74 \pm 0.93\ \mu$M), demonstrating that the additional iron bound to transferrin *in vivo* can be entirely explained by free iron initially present in the Hb–DBBF transfusate. The 25 μM readily chelatable iron in the Hb–DBBF solutions used here probably represent a subset of the total labile iron, calculated to be approx. 80 μM in solutions of Hb–DBBF [43].

The other EPR signals illustrated in Figure 2(a), ceruloplasmin, catalase and the erythrocyte globin radical(s), did not significantly change during the transfusion process, nor for the subsequent 3 h. No significant changes were seen in any physiological or EPR parameters in the control animals, following the re-transfusion of autologous blood (Table 1).

Thus the addition of Hb–DBBF to an animal adds two forms of iron that can potentially initiate free radical damage. The free iron (5 μM) can be effectively detoxified via binding to transferrin. However, the methaemoglobin 'load' (50 μM) would be expected, if anything, to increase with time owing to autoxidation of the remaining 95 % of oxyhaemoglobin. However, Table 1 shows that the EPR-detected methaemoglobin concentration in plasma decreases with time following the transfusion with a $t_{1/2}$ of approx. 1 h. This decrease must arise from a reductive, degradative or transport process. A degradative process would be expected to cause an increase in iron bound to transferrin with time which was not seen; a transport process (actively removing metHb–DBBF from the plasma) seems unlikely: total Hb–DBBF remained unchanged over the relatively short time course of the study and the transport system would have to specifically target methaemoglobin, which seems structurally implausible. Therefore we view the most likely explanation as a reductive process in the plasma, converting the metHb–DBBF into oxyHb–DBBF.

The principal aqueous low-molecular-mass reducing agents in plasma are urate, glutathione and ascorbate. As implied by earlier work on HbA_o [44], *in vitro* experiments failed to demonstrate any significant reduction of metHb–DBBF by glutathione or urate (results not shown). Ascorbate, however, is able to reduce metHb–DBBF to oxyHb–DBBF (Figure 3a), but at a rather low rate. The second-order rate constant (at 37 °C in PBS, pH 7.4) was calculated to be $0.2\ M^{-1} \cdot s^{-1}$. This rate requires non-physiological plasma ascorbate concentrations (in excess of 500 μM) to be able to explain the observed reduction of methaemoglobin *in vivo* seen in Table 1. However, the presence of erythrocytes appeared to increase the efficiency of ascorbate reduction of metHb–DBBF (Figure 3b). The effect required whole cells, and could not be reproduced by erythrocyte membranes (ghosts) alone (Figure 3c). This agrees with previous findings that the role of the erythrocyte in catalysing extracellular methaemoglobin reduction by ascorbate is due to the recycling of dehydroascorbate to ascorbate, rather than a specific erythrocyte membrane enzyme [45,46]. Erythrocytes alone had a negligible ability to reduce metHb–DBBF, in the absence of exogenous ascorbate (Figure 3d).

Detailed analysis of the plasma EPR spectra before and after transfusion revealed an anomalous finding (Figure 4). The only change in the $g = 2$ region related to the features attributable to the high-field components of the methaemoglobin EPR spectrum (at $g = 1.99$). When these features are removed (via subtracting an appropriate pure haemoglobin spectrum), the remaining spectrum only contained features attributable to the copper centres in ceruloplasmin. In contrast with the situation inside the erythrocyte, where the globin free radical can clearly be seen (Figure 2), we see no detectable protein free radical bound to Hb–DBBF in the plasma. This is despite higher concentrations of metHb–DBBF in the plasma (compared with the erythrocyte) and, presumably, significant peroxide formation due to autoxidation of Hb–DBBF and/or other oxidative stress mechanisms [47].

The difference cannot be due to an intrinsic difference in Hb–DBBF compared with HbA_o. Both have similar autoxidation rates (Hb–DBBF is slightly higher than HbA_o [48]) and both form similar radicals *in vitro* following peroxide addition. Therefore we tested whether plasma ascorbate could scavenge the H_2O_2-induced free radical on Hb–DBBF. Figure 5 shows that the free radical formed on pure Hb–DBBF by H_2O_2 becomes undetectable in the presence of ascorbate. Instead a spectrum with identical features with those of the ascorbyl radical is observed. In low-temperature experiments (Figure 5a), this is characterized by a narrow 9 G ($1\ G = 10^{-4}\ T$) singlet with anomalous power saturation characteristics [49]; at room temperature (Figure 5b), a doublet is observed [50]. The only detectable EPR radical signal formed following H_2O_2 addition to metHb–DBBF in plasma has identical features with that of the ascorbyl radical (Figure 5c). Removal of plasma ascorbate via pre-treatment with ascorbate oxidase resulted in the appearance of non-ascorbyl radicals (Figure 5d); the exact species responsible for this symmetrical radical ($g = 2.004$) is not clear. However, it is inconsistent with low-molecular-mass urate or glutathione-based radicals. This confirms that it is the presence of ascorbate in plasma that makes it impossible to observe protein radical EPR spectra in the rabbit plasma following transfusion.

Plasma ascorbate also has the potential to reduce the other oxidizing species formed following H_2O_2 addition to methaemoglobin, ferryl iron [51]. Figure 6 demonstrates that the addition of ascorbate to ferrylHb–DBBF causes a rapid formation of metHb–DBBF. This reactivity exhibits saturating behaviour with respect to ascorbate with an observed V_{max} of $\sim 50\ s^{-1}$ (results not shown). Subsequently, metHb–DBBF is converted into oxyHb–DBBF by the slow process described previously (Figure 3). In the steady state, ascorbate keeps the concentration of ferryl iron in globins at levels that are essentially undetectable optically [52,53]; this effect is mediated solely by an effect on ferryl reduction, as ascorbate has no effect on the rate constant for ferryl formation from H_2O_2 and metHb–DBBF (results not shown).

Ferryl iron and globin free radicals can react together to induce oxidative modifications of the haem that are detectable in normal human blood and are increased under conditions of oxidative stress [47]. More severe oxidative conditions (requiring high peroxide or lowered pH) result in a covalent haem-to-protein bond [2,10,48,54]. This species (Hb-H) is indicative of enhanced ferric:peroxide reactivity *in vivo* [15]. Figure 7 shows that physiological levels of ascorbate decrease the formation of Hb-H *in vitro*. This is even the case at acid pH, where the reactivity of the ferryl iron is at its greatest *in vitro* and *in vivo*. We were unable to detect Hb-H *in vivo* in the control or Hb–DBBF-transfused animals. Thus the normal physiological defence mechanisms are able to prevent Hb-H formation following a 20 % transfusion of Hb–DBBF, unlike the case when haem is released outside the erythrocyte in certain disease states, e.g. rhabdomyolysis [13,14]

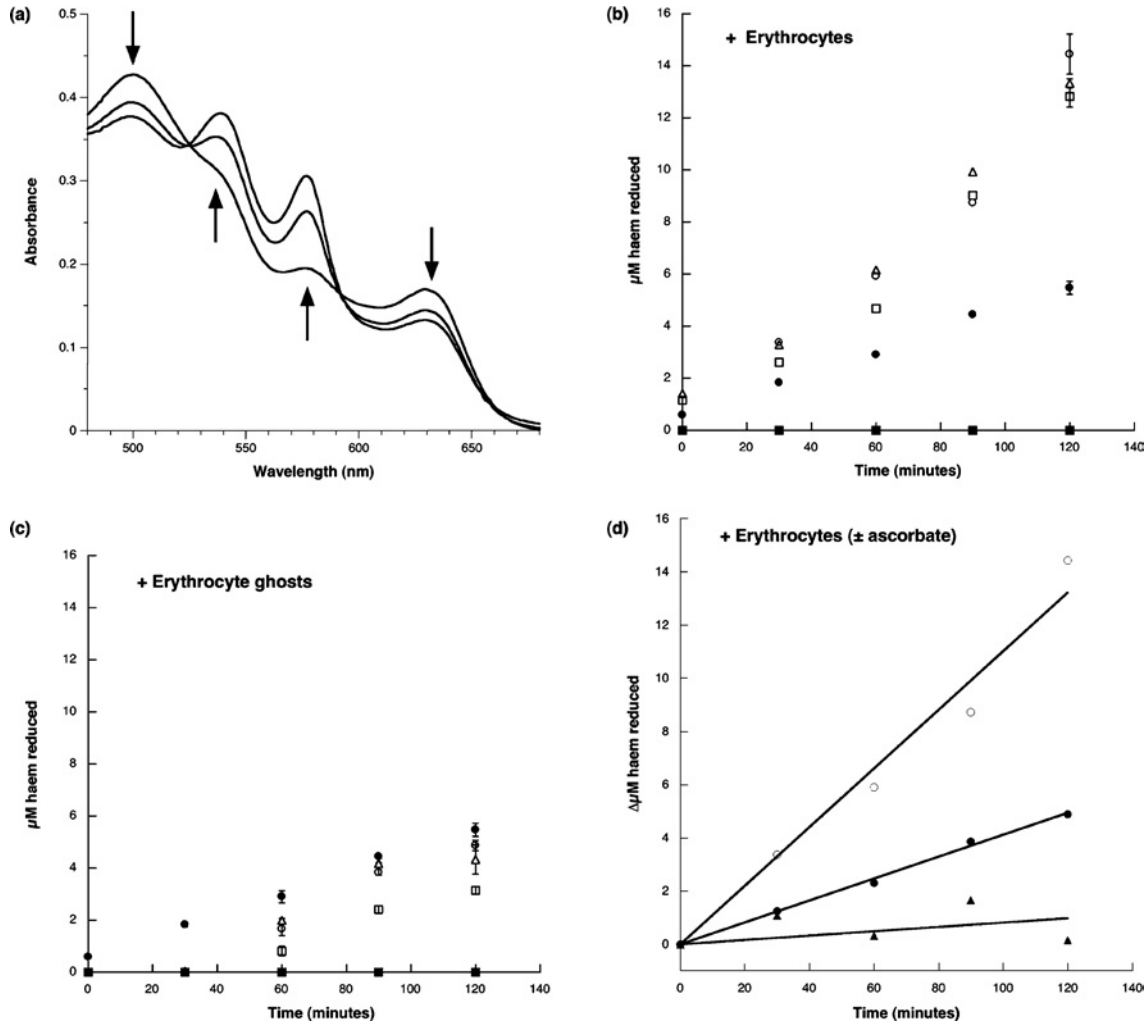

Figure 3 Reduction of metHb–DBBF to oxyHb–DBBF by ascorbate *in vitro* and *in vivo*

(**a**) Addition of ascorbate (1.5 mM) to metHb–DBBF (50 μM) in PBS at 37 °C. Lines illustrate spectra before addition and 30 and 60 min after addition. Arrows indicate change in absorbance with time. (**b**) Reduction of 100 μM metHb–DBBF by 100 μM ascorbate in the presence of various concentrations of erythrocytes. Control (100 μM metHb–DBBF alone) (■); + ascorbate alone (●); ascorbate + 20 % haematocrit (△); ascorbate + 30 % haematocrit (□); ascorbate + 40 % haematocrit (○). Results are means ± S.D. ($n = 3$), but note that, except at greater time points, the errors are less than 2.5 % of the mean, thus error bars are generally not visible within the Figure. (**c**) Reduction of 100 μM metHb–DBBF by 100 μM ascorbate in the presence of various concentration of erythrocyte ghosts. Results are as in (**b**), except that ghosts were used rather than erythrocytes. Statistical analysis (see the Experimental section) revealed that ascorbate significantly increased metHb reduction and this was enhanced by the addition of erythrocytes (but not ghosts). There was no difference between the effect of 20 %, 30 % or 40 % haematocrit. (**d**) Synergistic effects of ascorbate and erythrocytes on the reduction of 100 μM metHb–DBBF. Change in oxyHb–DBBF concentration with time: 40 % haematocrit alone (▲); 100 μM ascorbate alone (●); 100 μM ascorbate + 40 % haematocrit (○).

or subarachnoid haemorrhage [15]. The chemical reactivity of ascorbate described here is therefore sufficient, in and of itself, to explain all of the plasma defences to haem-protein-mediated oxidative stress.

DISCUSSION

The present paper reports on the effect of infusing a high dose of a haem protein into plasma. In particular, we have focused on the haem redox chemistry as we have shown previously that this is responsible for the kidney damage due to massive myoglobin release in rhabdomyolysis [14]. Haem-mediated oxidative side reactions of cell-free haemoglobin have emerged as an important pathway of toxicity, which may have possibly contributed to recent clinical setbacks with the use of haemoglobin-based products as blood substitutes [16].

The spectroscopic approach adopted informs both on the haemoglobin itself and, directly and indirectly, on the response of the plasma antioxidant defences to the 'haem insult'. We show that, in the presence of peroxide, Hb–DBBF undergoes an identical stoichiometric conversion from the ferric into the ferryl redox state as occurs with HbA_o. H_2O_2 accepts two electrons from ferric haemoglobin (see eqn 1). One converts ferric into ferryl, and the other results in the formation of a cationic radical. The nature of this radical is complex. Unlike the situation with peroxidases and catalases that are designed to react with H_2O_2, and maintain the radical in a controlled environment to catalyse subsequent oxidation reactions [5], the radical formed in the 'pseudoperoxidase' activity of myoglobin and haemoglobin is less stable and resides on tyrosine and tryptophan amino acids in the globin polypeptide chain [27,33–35,55]. In the present paper, we report that the EPR spectrum of Hb–DBBF following H_2O_2 addition is identical with that seen with HbA_o. Both the tyrosyl and the tryptophan peroxyl radicals can be detected, confirming

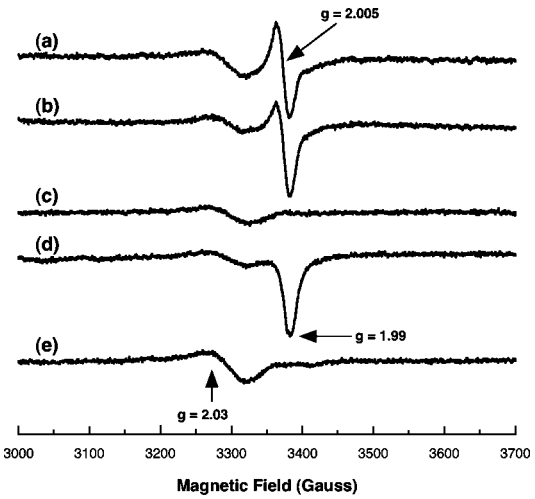

Figure 4 Free radicals in blood and plasma before and after Hb–DBBF transfusion

Illustrative EPR spectra from venous blood and venous plasma in a rabbit transfused with Hb–DBBF: (**a**) venous blood before transfusion; (**b**) venous blood immediately after transfusion; (**c**) venous plasma before transfusion; (**d**) venous plasma immediately after transfusion; (**e**) venous plasma with spectral contribution of methaemoglobin at $g = 1.99$ removed by subtraction of appropriate concentration of pure methaemoglobin spectrum. The g values indicated are those of ceruloplasmin ($g = 2.03$), the globin free radical ($g = 2.005$) and the low-field feature of methaemoglobin ($g = 1.99$). EPR conditions: temperature, 10 K; microwave power, 3.18 mW; microwave frequency, 9.47 GHz; modulation frequency, 100 kHz; modulation amplitude, 3 G; number of scans, 2; gain, 1×10^5; time constant, 40.96 ms; scan rate, 5.9 G/s.

that the cross-linking of α-subunits of Hb–DBBF (engineered to keep the protein in the circulation) do not affect the nature of the redox chemistry at the haem site, nor the subsequent radical migration.

The EPR spectrum of whole blood allows us to measure the ferric redox state of haemoglobin *in vivo* ($g = 6$ peak). The erythrocyte (catalase) and plasma (ceruloplasmin) antioxidant enzymes also have EPR-detectable ferric iron and cupric copper respectively, allowing quantitative measures of these enzyme concentrations to be determined. Apotransferrin is EPR-silent, but, once iron binds, a distinct EPR signal appears, allowing the release of iron into the plasma to be monitored. Finally, and most importantly, the free radical bound to globin (and produced via the spontaneous reaction of peroxides with methaemoglobin) can also be detected inside the erythrocytes. A single EPR spectrum can therefore simultaneously and quantitatively inform on all significant paramagnetic species in the blood.

What are the changes in the paramagnetic species in blood following a 20 % transfusion of a haemoglobin-based blood substitute? The clearest change is the increase in the methaemoglobin signal. We have shown that this is entirely due to metHb–DBBF in the plasma and pre-existing in the solution before transfusion. This level (5 %) is within the normal acceptable range for a blood substitute and results from the balance between the rate of reduction and autoxidation during its manufacture and storage [43]. Nevertheless, even this level of methaemoglobin has the potential to catalyse oxidative damage. In addition to the change in methaemoglobin, EPR demonstrated that there was an increase in iron binding to transferrin consistent with a 25 μM concentration of loosely bound iron in the Hb–DBBF pre-infusion. Following the 20 % transfusion, the 5 μM 'free iron' was rapidly bound to transferrin, where it became redox-inactive. The level of iron binding to transferrin did not increase during the course of the study, and remained well below full saturation. Iron was not released from Hb–DBBF to any significant extent in the 3 h period after transfusion. Therefore Fenton chemistry (where free ferrous iron reacts with H_2O_2 to form the strongly pro-oxidative hydroxyl radical, $OH^{\bullet}$) is highly unlikely to play a direct role in the plasma following haem protein release, either in spontaneous haemolysis, haemolytic diseases or following the infusion of a haemoglobin-based blood substitute.

The most significant finding of the present study is, paradoxically, one relating to the absence of a free radical EPR signal. Throughout the study, the globin radical in the erythrocyte remained at a constant concentration in both the control and Hb–DBBF-transfused animals. However, no free radical was present in the plasma following Hb–DBBF treatment, despite the fact that we know that Hb–DBBF makes identical (and therefore readily detectable) radicals with those of HbA_o. The absence of any spectral features attributable to such a sharp feature in the EPR spectrum implies that the concentration must be held at less than 5 nM. We demonstrated that this was entirely consistent with the ability of plasma concentrations of ascorbate to scavenge this radical. Furthermore, ascorbate had the ability to directly reduce the ferrylHb–DBBF, in agreement with previous studies on HbA_o and myoglobin [51]. Ascorbate is therefore able to chemically reduce both of the toxic oxidants formed following peroxide addition to erythrocyte-free haemoglobin (described in eqn 1).

Ascorbate not only has the ability to deal with the oxidative products of eqn 1, but also can decrease the concentration of the substrate, methaemoglobin [56]. However, unlike the situation with the oxidative products, this does not involve simple chemistry, but requires the presence of the erythrocyte. As Hb–DBBF cannot readily enter the erythrocyte, this suggests the possibility of a methaemoglobin reductase system acting at the external face of the erythrocyte membrane, ultimately driven by electrons from plasma ascorbate. Trans-plasma membrane reductase systems in the erythrocyte have been characterized [57,58]. These redox pathways are able to take electrons from intracellular ascorbate (or NADH) and reduce external ferricyanide [59]. However, methaemoglobin itself cannot be reduced directly by this system. Our studies reveal that erythrocyte ghosts do not enhance metHb–DBBF reduction, while earlier work demonstrated that reduction occurs even when a dialysis membrane separates the protein and the erythrocyte solutions [45].

Recent studies have suggested that the physiological electron acceptor for the trans-plasma membrane reductase is in fact the ascorbyl radical [60,61]. This appears to be a high-capacity system: up to 35 μM external ascorbate can be regenerated in blood every 3 min [46]. The activity of this system would therefore be more than enough to maintain a constant steady-state extracellular ascorbate concentration in our *in vitro* study (Figure 3b). This relatively high rate of ascorbate regeneration, followed by the rate-limiting step of ascorbate oxidation by methaemoglobin, also explains why increasing the haematocrit above 20 % had no effect in our study; as even this low haematocrit should be sufficient to generate 30 μM external ascorbate per h [61]. It also explains why in the plasma we did not see high concentrations of the ascorbyl radical, despite our prediction of significant ascorbate oxidation by metHb–DBBF.

The most likely role for the erythrocyte in defending against blood-substitute-induced methaemoglobinaemia is therefore to maintain a constant plasma ascorbate concentration despite the presence of high concentrations of extracellular methaemoglobin. The reduction of methaemoglobin by ascorbate is linearly dependent on ascorbate concentration and any significant decrease in plasma ascorbate would significantly decrease this rate. Extrapolating from *in vitro* reduction rates to *in vivo* methaemoglobin

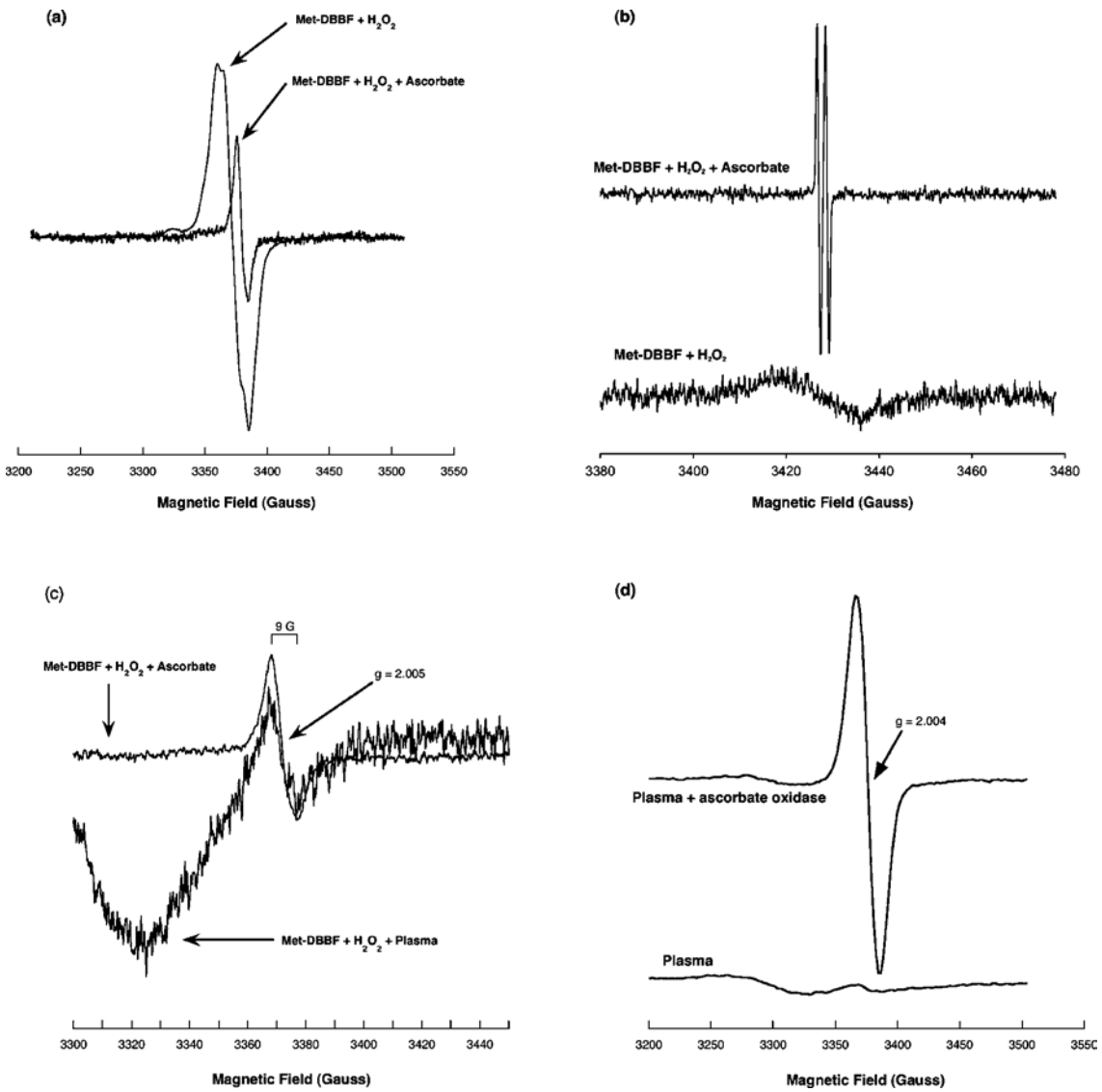

Figure 5 Effects of ascorbate on haemoglobin radicals

(**a**) EPR spectra 30 s after addition of H_2O_2 (100 μM) to metHb–DBBF (50 μM) in PBS at 37 °C in the presence or absence of sodium ascorbate (200 μM), added immediately before the peroxide. Ascorbate spectrum illustrated at 5× magnification. (**b**) As in (**a**), but the experiment measured at room temperature. (**c**) EPR spectrum of rabbit plasma following addition of metHb–DBBF (50 μM) and H_2O_2 (100 μM); the sample was frozen 30 s after peroxide addition. Overlaid with spectrum from (**a**) of peroxide addition to metHb–DBBF in the presence of ascorbate. Plasma spectrum illustrated at 5× magnification. (**d**) As (**c**), but plasma pre-incubated for 30 min with ascorbate oxidase (15 units from *Cucurbita* sp.; Sigma Chemical Co.). Any contributions of the methaemoglobin $g = 1.99$ signal to low-temperature studies were removed by subtraction of appropriate concentrations of the pure methaemoglobin spectrum. EPR conditions (**a**, **c** and **d**): temperature, 10 K; microwave power, 50 μW; microwave frequency, 9.47 GHz; modulation amplitude, 3 G; time constant, 81.92 ms; scan rate, 3.6 G/s. EPR conditions (**b**): temperature, 295 K; microwave frequency, 9.62 GHz; microwave power, 2 mW; modulation amplitude, 1 G; scan rate, 1.2 G/s; time constant, 81.92 ms.

levels is not trivial, e.g. oxyHb–DBBF will be continually regenerating metHb–DBBF due to autoxidation. However, the data suggest simple reduction by plasma ascorbate as being a dominant mechanism. This role of ascorbate may go beyond the problems of extracellular methaemoglobin in blood substitutes. For example, the increase in methaemoglobin formation following decreases in ascorbate levels after air blast overpressure injury is also consistent with a role for ascorbate in preventing methaemoglobinaemia [62].

How does ascorbate compare with other plasma antioxidants (e.g. urate and glutathione) in dealing with haem-protein mediated oxidative stress? We propose three roles for ascorbate. Reduction of exogenous methaemoglobin, reduction of ferryl and globin free radical scavenging. The reduction of methaemoglobin cannot be catalysed by glutathione or urate and is unique to ascorbate. However, urate does have the potential to contribute to ferryl and protein radical scavenging. Urate is a good radical scavenger and can scavenge globin radicals [63]. Urate is also able to reduce ferryl [64]. The published rate constants, however, suggest that at similar concentrations ascorbate outperforms urate [65]. We performed studies with ferrylHb–DBBF at the approximate concentrations present in rabbit plasma (50 μM ascorbate and 30 μM urate) and confirmed that ascorbate is a kinetically more competent ferryl reductant under these conditions (results not shown).

One other reaction that probably contributes to ferryl reduction is electron transfer from ferrous haemoglobin [65]. Although slower than reduction by either ascorbate or urate, the higher

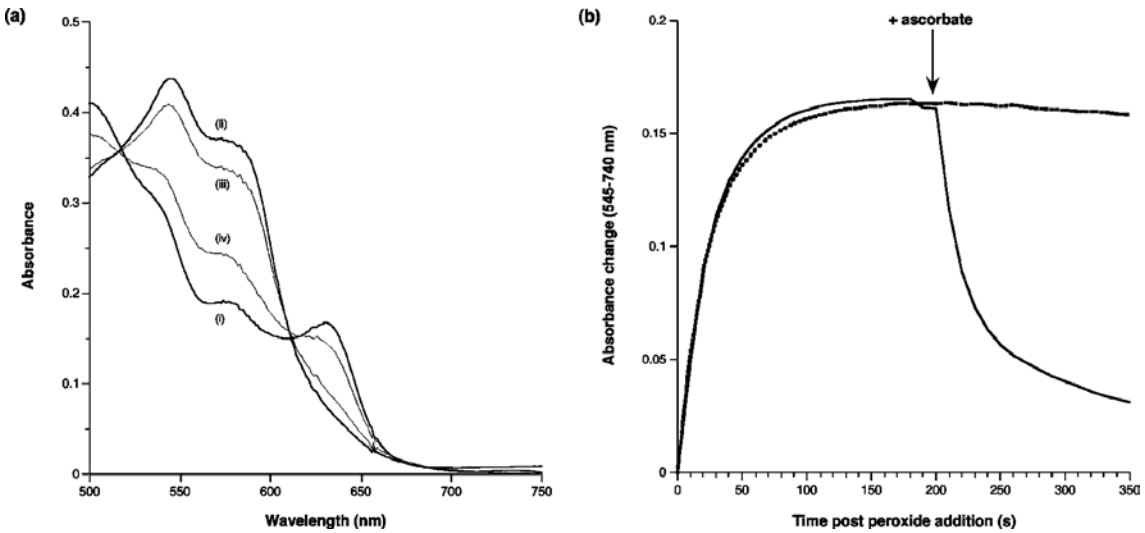

Figure 6 Effects of ascorbate on ferryl haemoglobin

Optical spectral changes following H_2O_2 and ascorbate addition to metHb–DBBF. (**a**) H_2O_2 (50 μM) was added to metHb–DBBF (50 μM) in PBS at 37 °C. (i) metHb–DBBF, (ii) metHb–DBBF 2 min after H_2O_2 addition, (iii) metHb–DBBF 10 s after the addition of 1.5 mM sodium ascorbate, (iv) metHb–DBBF 50 s after ascorbate addition. (**b**) Change in absorbance at 545 nm following H_2O_2 addition to metHb–DBBF (50 μM) in PBS at 37 °C (dotted line). In the experiment illustrated by the solid line, 1.5 mM sodium ascorbate was added at the point of maximum ferryl formation.

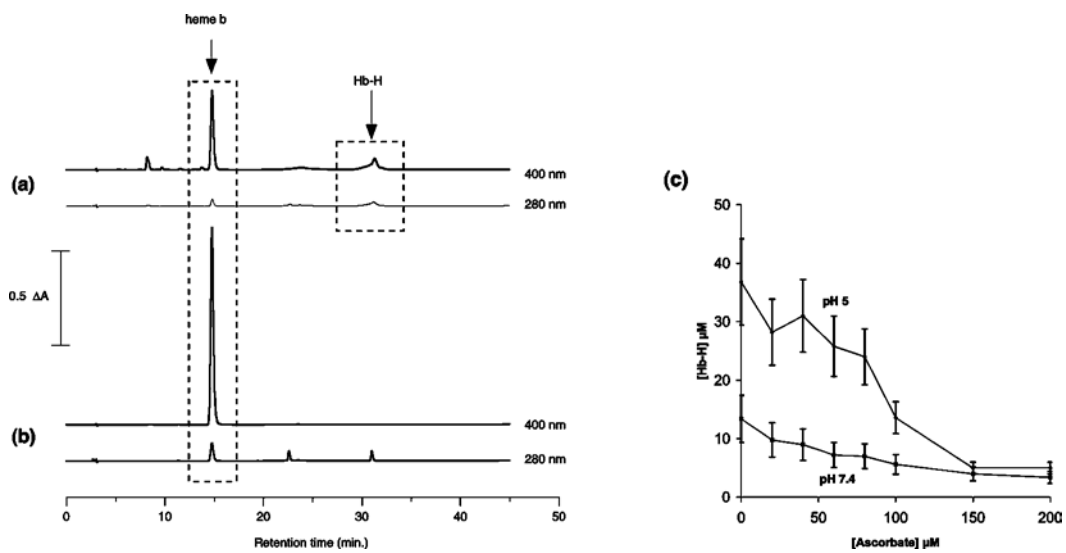

Figure 7 Effects of ascorbate on peroxidative damage to haemoglobin

Reverse-phase HPLC chromatogram of metHb–DBBF (100 μM) reacted with H_2O_2 (200 μM) for 1 h at 37 °C in sodium acetate (20 mM), pH 5.0, (**a**) in the absence and (**b**) in the presence of 0.6 mM sodium ascorbate. The peak at 15 min is free haem *b* (visible at both 400 nm and 280 nm detection). The peaks at 22.7 and 31 min are from the β- and modified α-chains in Hb–DBBF. Normally, these are only detectable at 280 nm, but, in the absence of ascorbate, there is significant absorbance at 400 nm, indicating a covalent haem:protein attachment that cannot be disrupted at the pH of the HPLC column (pH 2). The optical spectrum of this species (not shown) is that previously ascribed to this covalent haem:protein species following peroxide addition to HbA_0 (termed Hb-H). (**c**) Effect of varying the ascorbate concentration on the formation of Hb-H at pH 5.0 (in 20 mM sodium acetate) and pH 7.4 (in 20 mM sodium phosphate) at 37 °C.

concentrations of deoxyHb–DBBF *in vivo* are likely to make this a major reaction at higher transfusion levels. However, the products of this comproportionation reaction are two molecules of metHb–DBBF, making the role of ascorbate in reducing methaemoglobin even more important.

The studies following ascorbate oxidase addition to rabbit plasma demonstrate that ascorbate is the primary low-molecular-mass antioxidant when dealing with aqueous radicals in rabbit plasma. We were not able to identify low-molecular-mass thiyl or urate [63] radicals in this system. However, we caution against a simple translation of these findings to the human system. Urate levels [64] are significantly higher in primates than rodents (reaching up to 300 μM); although ascorbate is still the dominant scavenger of aqueous radicals in human plasma [66], at these levels, urate is more effective than ascorbate at scavenging ferryl haemoglobin ([65], and results not shown).

The proposed multifunctional role of ascorbate in limiting the redox toxicity of haem proteins (as a radical scavenger and a ferric and ferryl haem iron reductant) would be expected to function irrespective of whether the haem protein was released into the plasma as part of normal physiology/pathophysiology, or following the infusion of a haemoglobin-based blood substitute.

However, the present study has specific implications for the clinical use of blood substitutes: (i) the co-administration of reducing agents, such as ascorbate, may offer a simple strategy to control or reduce haemoglobin-mediated toxicity; and (ii) the haematocrit should not be dropped too low as, even if oxygen transport is adequate, the decrease in erythrocyte concentration could compromise methaemoglobin reduction.

We acknowledge funding from a Wellcome Trust Biomedical Research Collaboration Grant (to C.E.C. and P.M.), a BBSRC studentship (to J.D.) and the European Union STREP (Specific Targeted Research Projects) 'EUROBLOODSUBSTITUTES' (to C.E.C., M.T.W., P.M. and R.S.-D.). We thank Dr Dimitri Svistunenko (University of Essex) for assistance with the interpretation of EPR spectra. The opinions and assertions contained herein are the scientific views of A.I.A. and are not to be construed as policy of the U.S. Food and Drug Administration.

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Received 2 March 2006/19 June 2006; accepted 18 July 2006
Published as BJ Immediate Publication 18 July 2006, doi:10.1042/BJ20060341