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LABORATORY INVESTIGATIONS

Effect of propofol and thiopentone on free radical mediated oxidative stress of the erythrocyte†

P. G. MURPHY, M. J. DAVIES, M. O. COLUMB AND N. STRATFORD

Summary

Propofol has free radical scavenging properties similar to those of recognized phenol-based antioxidants. We have examined these properties in an *in vitro* model of radical-induced cellular injury, comparing its activity with that of thiopentone (which has also been shown to have radical scavenging activity). Haemolysis of human erythrocytes was induced using the azo compound 2,2′-azo-bis(2-amidinopropane) dihydrochloride (ABAP). This was achieved by incubating a 10 % suspension of erythrocytes with ABAP 100 mmol litre^{-1} at 37 °C. For propofol, at concentrations of 12.5, 25 and 50 μmol litre^{-1}, the times to achieve 50 % haemolysis were mean 126 (SEM 7) min (95 % confidence interval 108–144 min), 150 (8) (129–170) min and 182 (12) (160–180) min, respectively (Intralipid control 107 (7) (90–125) min, ANOVA $P < 0.0001$). For thiopentone, at concentrations of 62.5, 125 and 250 μmol litre^{-1}, the values were 117 (2) (112–121) min, 126 (3) (119–133) min and 138 (2) (132–144) min, respectively (saline control 109 (2) (104–113) min, ANOVA $P < 0.0001$). Spectroscopic analysis in the visible and ultraviolet spectra demonstrated a steady increase in the proportion of methaemoglobin during haemolysis, with the highest concentrations in the propofol-containing flasks. The formation of methaemoglobin was preceded by the generation of ferrylhaemoglobin (a Fe^{4+} haemoglobin species). Further experiments examining oxidation of purified methaemoglobin to ferrylhaemoglobin by hydrogen peroxide suggested that propofol, but not Intralipid or thiopentone, reduced ferrylhaemoglobin back to the met- state, and thereby explained the higher concentrations of methaemoglobin in the propofol-containing erythrocyte suspensions. We conclude that propofol is a more potent free radical scavenger in this model of oxidant stress than thiopentone, and that reduction of high oxidation states of haemoglobin may contribute to such activity. (*Br. J. Anaesth*. 1996; **76**: 536–543)

Key words

Anaesthetics i.v., propofol. Anaesthetics i.v., thiopentone. Blood, haemoglobin. Blood, erythrocytes. Blood, haemolysis.

Membrane peroxidation is regarded as a major mechanism whereby free radicals damage biological tissue [1]. Radical-mediated peroxidation of erythrocyte membranes leads to haemolysis and can be induced by a variety of reactive oxygen species, such as hydrogen peroxide [2] superoxide anion [3] and peroxyl radicals derived from the thermal decomposition of water-soluble and lipid-soluble azo compounds [4, 5]. The latter compounds provide an easily reproducible and reliable model of radical-induced haemolysis [4] and have demonstrated the importance of the endogenous lipid-soluble free radical scavenger α-tocopherol (vitamin E) in protecting the erythrocyte membrane against such injury [6]. The intracellular contents of the erythrocyte may also be susceptible to oxidant stress. For example, the haem-based iron of ferrous haemoglobin (Hb-Fe^{2+}) undergoes electron transfer reactions to form higher oxidation states such as met- (Hb-Fe^{3+}) and ferryl- (Hb-Fe^{4+}) haemoglobin species:

$$\text{Hb-Fe}^{2+} \leftrightarrow \text{Hb-Fe}^{3+} + e^- \leftrightarrow \text{Hb-Fe}^{4+} + e^- \quad (1)$$

The formation of higher oxidation states of haemoglobin has been described when erythrocytes are exposed to superoxide anion [3] and hydrogen peroxide [3, 7], although not when haemolysis is initiated by azo compounds [6].

The anaesthetic agent 2,6-diisopropylphenol is a potent antioxidant, chemically similar to α-tocopherol [8–10], and as such is a potent inhibitor of lipid peroxidation [8, 9]. In the *in vitro* experiments reported here, we have examined the effect of the clinical formulation of 2,6-diisopropylphenol, that is propofol, on peroxidation of erythrocytes induced by the water-soluble azo compound 2,2′-azo-bis(2-amidinopropane) dihydrochloride (ABAP). This has been evaluated in terms of haemolysis (as a measure of membrane integrity) and the haemoglobin redox state (as a measure of intracellular oxidant stress). The latter experiments showed that considerable quantities of met- and ferrylhaemoglobin are formed during ABAP-induced haemolysis, and that propofol significantly interferes with such redox reactions. In

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order to characterize this hitherto unreported property, we have examined the effect of propofol on the oxidation of purified methaemoglobin by hydrogen peroxide. As thiopentone has also been shown to have antioxidant properties (inhibiting lipid peroxidation *in vitro* [11]), we have also examined the relative activity of these two anaesthetic agents in this model of oxidant-induced cell injury.

Materials and methods

HAEMOLYSIS EXPERIMENTS

Preparation of RBC suspensions

Heparinized blood obtained on the day of experiment from six healthy, non-smoking volunteers was used for these studies (age range 25–35 yr, four males). After separation from plasma and buffy coat by centrifugation for 5 min at 4000 rpm, erythrocytes were washed three times with sterile NaCl 0.15 mmol litre^{-1}. During the last wash the cells were centrifuged for exactly 10 min at 4000 rpm to ensure a constant packed cell volume. The cells were then resuspended in four volumes of phosphate-buffered saline (PBS, NaCl 125 mmol litre^{-1}, Na_2HPO_4 10 mmol litre^{-1}, pH 7.4) to prepare a stock 20 % RBC suspension.

Induction of free radical-mediated haemolysis

Free radical-mediated haemolysis of the RBC suspensions was initiated with ABAP (Polysciences). ABAP (R–N=N–R) is a water-soluble azo compound which although stable at 4 °C decomposes at 37 °C to produce organic radicals (R·, reaction 2) at a steady rate:

$$R{-}N{=}N{-}R \rightarrow 2R\cdot + N_2 \qquad (2)$$

These radicals react with molecular oxygen to produce organic peroxyl radicals (RO_2· ; reaction 3)

$$R\cdot + O_2 \rightarrow RO_2\cdot \qquad (3)$$

which initiate peroxidative damage to membrane proteins and lipids, and thereby induce haemolysis. For example, organic peroxyl radicals react with unsaturated lipid (LH) to produce lipid radicals (L·) and organic hydroperoxides (ROOH):

$$RO_2\cdot + LH \rightarrow ROOH + L\cdot \qquad (4)$$

In our experiments haemolysis was induced by mixing equal volumes of ABAP (200 mmol litre^{-1} dissolved in PBS) and 20 % RBC suspensions to produce 10 % RBC suspensions in ABAP 100 mmol litre^{-1}. The suspensions were incubated at 37 °C under aerobic conditions and agitated gently throughout. Aliquots were obtained every 30 min for measurement of percentage haemolysis and spectroscopic analysis.

Measurement of haemolysis

The extent of haemolysis in the RBC suspensions was measured spectrophotometrically, by comparing the extracellular haemoglobin content of the aliquots with that of a fully haemolysed reference sample. Thus, 200 μl of RBC suspension was added to 3.8 ml of PBS and centrifuged for 4 min at 4000 rpm. The supernatant was added to an equal volume of Drabkin's reagent (stock solution, potassium ferricyanide 1.21 mmol litre^{-1}, potassium cyanide 1.54 mmol litre^{-1}, potassium dihydrogen phosphate 2.06 mmol litre^{-1}) in order to convert the various species of haemoglobin present to cyanmethaemoglobin, its most stable form. The absorption of this solution was measured at 540 nm. The fully haemolysed reference was prepared in the same way, except that the 200-μl aliquot was added to distilled water rather than PBS. Percentage haemolysis was measured according to the equation

$$\%\ \text{haemolysis} = A/B \times 100$$

where A = absorbance of the sample aliquot and B = absorbance of the fully haemolysed reference. A line plot of percentage haemolysis *vs* time was constructed, from which was estimated the time taken to achieve 50 % haemolysis (H_{50}, min).

Experimental design

The effect of propofol (Diprivan, Zeneca) was examined at 2, 6-diisopropylphenol concentrations of 12.5, 25 and 50 μmol litre^{-1}. The Intralipid content of the 12.5- and 25-μmol litre^{-1} flasks was adjusted to equal that of the 50-μmol litre^{-1} flask, as was that of the Intralipid control flask. The effect of thiopentone was examined at 62.5, 125 and 250 μmol litre^{-1}, with the equivalent control containing PBS. In both cases, the agents were added to the 200-mmol litre^{-1} stock solution of ABAP immediately before mixing with the 20 % RBC suspension. In order to exclude spontaneous haemolysis during the incubation period, a second (blank) control consisted of a 10 % RBC suspension with propofol or thiopentone but without ABAP.

Spectroscopic analysis of RBC suspensions

Different haemoglobin species have different absorption spectra and can therefore be distinguished spectroscopically. We investigated qualitatively and quantitatively the changes in the proportions of various haemoglobin species present during haemolysis. Qualitative assessments were made from absorption scans, measured using a Pye Unicam SP8-500 scanning spectrophotometer (range 500-700 nm, scan speed 2 nm s^{-1}). The proportions of the various haemoglobin species present in the reaction mixtures were made by measuring the absorbance of the suspensions at set wavelengths (560, 577 and 630 nm), correcting for background absorbance measured at 700 nm, and applying the following equations:

$$[\text{oxy-Hb}] = 0.068425A_{577} - 0.001442A_{560} - 0.082173A_{630}$$

$$[\text{met-Hb}] = 0.041828A_{577} - 0.079247A_{560} + 0.318075A_{630}$$

$$[\text{ferryl-Hb}] = 0.095969A_{560} - 0.054490A_{577} - 03046882A_{630}$$

where A = measured absorbance at each wavelength. These equations are similar to those described elsewhere for analysis of myoglobin [12] and were derived by solving the simultaneous equations for the measured absorbance at the given wavelengths set up using the following millimolar extinction coefficients for haemoglobin [13]: oxyhaemoglobin λ_{560} 8.6, λ_{577} 15.0, λ_{630} 0.17; methaemoglobin, λ_{560} 4.30, λ_{577} 4.45, λ_{630} 3.63; ferrylhaemoglobin, λ_{560} 14.1, λ_{577} 3.9, λ_{630} 3.0.

In further spectroscopic studies, we examined if this model of erythrocyte oxidative injury involved generation of transient intermediate ferrylhaemoglobin species such as Fe^{4+} = O-protein and Fe^{4+} = O, which are known to be generated during oxidation of methaemoglobin by peroxides. These unstable and reactive species are difficult to detect in cellular systems, particularly in low concentrations. The latter can be "trapped" by Na_2S, which reacts with ferrylhaem species to form stable oxysulpho compounds which absorb strongly at 620 nm [14]. Thus, in these experiments, ABAP-induced haemolysis was carried out in the presence of Na_2S 8 mmol litre^{-1}. The presence of oxysulphohaemoglobin was identified by spectral scanning and quantified by measuring the change in absorption at 620 nm.

HAEMOGLOBIN OXIDATION EXPERIMENTS

Spectroscopic analysis of the red cell suspensions showed that ABAP-induced haemolysis was associated with generation of both met- and ferrylhaemoglobin. Furthermore, there was consistently more methaemoglobin in the flasks containing propofol than those containing Intralipid, saline or thiopentone. In order to test the hypothesis that this latter observation was a result of the reduction of ferrylhaemoglobin (formed during ABAP-induced oxidative stress of the erythrocyte) back to methaemoglobin, we examined the effect of propofol on the peroxidation of methaemoglobin by hydrogen peroxide (H_2O_2).

Human haemoglobin (Sigma), which we found to be more than 80 % methaemoglobin, was prepared as a 23-μmol litre^{-1} solution in PBS. Precipitates were removed by centrifugation and the pH adjusted to 7.4 with NaOH. It was then oxidized with an excess of H_2O_2 (H_2O_2:haem of 20:1), to which was then added either propofol 12.5 or 50 μmol litre^{-1}, Intralipid, saline or thiopentone. All reactions were carried out at room temperature. Using the equations described above, the proportions of oxy-, met- and ferrylhaemoglobin were measured before and after the addition of H_2O_2 and at 10-min intervals for 30 min after the subsequent addition of propofol, thiopentone, Intralipid or saline.

STATISTICAL ANALYSIS

Values are expressed as mean (SEM) and 95 % confidence intervals. The effects of propofol and thiopentone on the rates of haemolysis, as expressed by H_{50} values, were analysed using analysis of variance (ANOVA), using Bartlett's test to determine homogeneity of variances of the groups [15]. Differences between individual groups were compared using a two-tailed unpaired *t* test, to which a Bonferroni correction was applied, and quantified by confidence interval analysis. The relationship between drug concentration and H_{50} values was explored further by applying a post-test for linear trend to the rank-ordered ANOVA tables [16]. This latter test describes the relationship of the measured variable (in this instance H_{50}) to column number in the ANOVA table, and thereby allows the underlying relationship between concentration of drug and H_{50} to be investigated. The effect of propofol on H_2O_2-induced peroxidation of methaemoglobin was analysed using ANOVA and Bonferroni corrected *t* tests.

Results

HAEMOLYSIS EXPERIMENTS

The effects of propofol and thiopentone on ABAP-induced haemolysis are shown in figure 1 and table 1. Propofol delayed ABAP-induced haemolysis in a concentration-dependent manner, and while at the concentration examined here complete haemolysis was eventually achieved, higher concentrations of propofol also suppressed the maximum degree of haemolysis observed (data not shown). Although thiopentone also delayed the onset of haemolysis, comparison of H_{50} values showed that its effect was less than that of propofol (table 1).

Spectroscopic analysis of the red cell suspensions

Spectral scanning of the reaction mixtures suggested a steady shift from oxy- to methaemoglobin during ABAP-induced haemolysis (fig. 2). A feature of this analysis was consistently greater absorption at 630 nm in the propofol-containing flask compared with that containing Intralipid. Quantitative spectroscopic analysis confirmed these findings (fig. 3). Thus there were significantly higher concentrations of methaemoglobin in the propofol flasks in comparison with the Intralipid-containing control. As

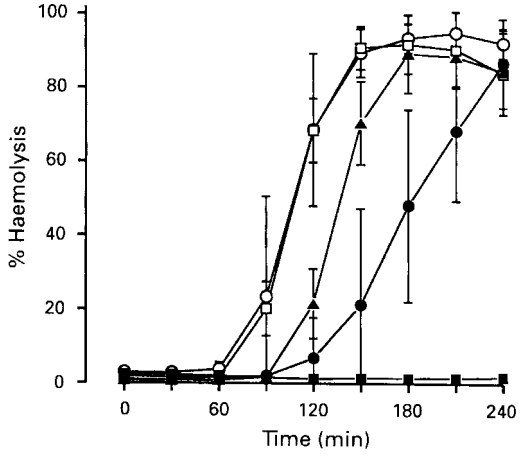

Figure 1 Effect of propofol 50 μmol litre^{-1} (●), Intralipid (□) and thiopentone 250 μmol litre^{-1} (▲) on ABAP-induced haemolysis (mean, 95 % confidence intervals, n = 6 in each case). ■ = Non-haemolysing control (erythrocyte suspension in PBS, no ABAP), ○ = haemolysing control.

Table 1 Effect of propofol and thiopentone on free radical-induced haemolysis. The rate of haemolysis is expressed as the time taken to achieve 50 % haemolysis (H_{50} (min), mean (SEM) (95 % confidence interval)). *ANOVA: $P < 0.0001$ (Bartlett's test for homogeneity of variances, $P = 0.57$). *t* test: Propofol 25 μmol litre^{-1}, *vs* Intralipid, $P < 0.05$; propofol 50 μmol litre^{-1} *vs* Intralipid, $P < 0.001$; propofol 12.5 *vs* 50 μmol litre^{-1}, $P < 0.01$. Test for linear trend: $r^2 = 0.6634$, $P < 0.0001$. †ANOVA: $P < 0.0001$ (Bartlett's test for homogeneity of Variances, $P = 0.73$). *t* test: Thiopentone 125 and 250 μmol litre^{-1} *vs* saline, $P < 0.001$; thiopentone 62.5 *vs* 125 μmol litre^{-1}, $P < 0.05$; thiopentone 62.5 *vs* 250 μmol litre^{-1}, $P < 0.001$; thiopentone 125 *vs* 250 μmol litre^{-1}, $P < 0.01$. Test for linear trend: $r^2 = 0.81$, $P < 0.0001$

	Intralipid	12.5 μmol litre^{-1}	25 μmol litre^{-1}	50 μmol litre^{-1}
Propofol*	107 (7) (90–125)	126 (7) (108–144)	150 (8) (129–170)	182 (12) (160–180)
	Saline	62.5 μmol litre^{-1}	125 μmol litre^{-1}	250 μmol litre^{-1}
Thiopentone†	109 (2) (104–113)	117 (2) (112–121)	126 (3) (119–133)	138 (2) (132–144)

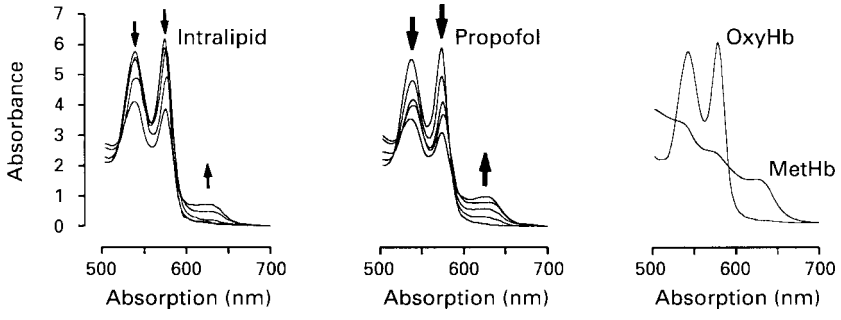

Figure 2 Sequential spectral absorption scans (absorption expressed in arbitrary units) of red cell suspensions containing Intralipid or propofol 50 μmol litre^{-1} during ABAP-induced haemolysis. Scans were performed every 60 min for 240 min and overlaid for ease of analysis. For comparison, the scans of pure oxy- (OxyHb) and methaemoglobin (MetHb) are shown. The direction of the changes in the sequential spectra are shown by the arrows. At time 0 both flasks contained predominantly oxyhaemoglobin. In the Intralipid flask there was little change before 180 min when the increase in absorption at 630 nm and the corresponding reduction in absorption at 540 and 577 nm suggest the formation of methaemoglobin. In comparison with Intralipid, the presence of propofol appeared to accelerate this transition from oxy- to methaemoglobin, with absorption at 630 nm being higher at any given time.

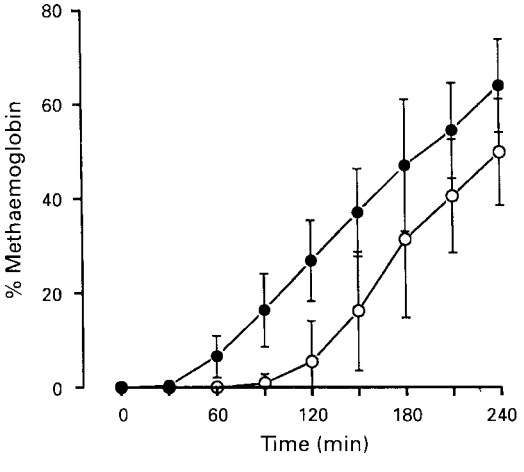

Figure 3 Effect of propofol 50 μmol litre^{-1} (●) and Intralipid (○) on the level of methaemoglobin measured by quantitative spectroscopic analysis during ABAP-induced haemolysis (expressed as percentage of total haem; mean, 95 % confidence intervals, $n = 6$). The effects of propofol 12.5 and 25 μmol litre^{-1} were intermediate between those of propofol 50 μmol litre^{-1} and Intralipid and were omitted for the sake of clarity. There was no difference between Intralipid and saline (the latter data were also omitted).

there was no difference between the concentrations of methaemoglobin in the Intralipid and saline flasks, this appeared to be an effect of propofol rather than Intralipid. Comparison of haemolysis and methaemoglobin data showed that while in the Intralipid-containing flasks methaemoglobin appeared only *after* cell lysis had started, in the propofol-containing flasks the appearance of methaemoglobin *preceded* haemolysis (fig. 4). In contrast with propofol, thiopentone had little effect of the concentrations of methaemoglobin (data not shown).

While confirming that oxidation of oxyhaemoglobin to the met- state had taken place, this quantitative analysis also suggested that in the early stages of haemolysis, 5–10 % of the total haemoglobin content was ferrylhaemoglobin (fig. 5). Furthermore, the proportion of this Fe^{4+} species was consistently lower in the presence of propofol in comparison with Intralipid. As relatively low concentrations of ferrylhaemoglobin were detected, it was felt important to obtain independent evidence for its generation during ABAP-induced haemolysis, using the sulphide-trapping method. Absorption at 620 nm (indicative of the reaction product of ferryl-

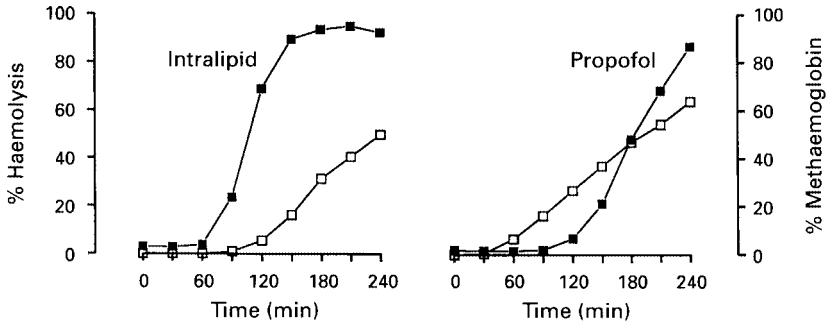

Figure 4 Effect of propofol 50 μmol litre^{-1} and Intralipid on the relationship between haemolysis (■) and increase in methaemoglobin (□) (mean, $n = 6$). In the Intralipid-containing flasks, haemolysis preceded significant methaemoglobin formation. In contrast, the presence of propofol, while delaying haemolysis, promoted the formation of methaemoglobin.

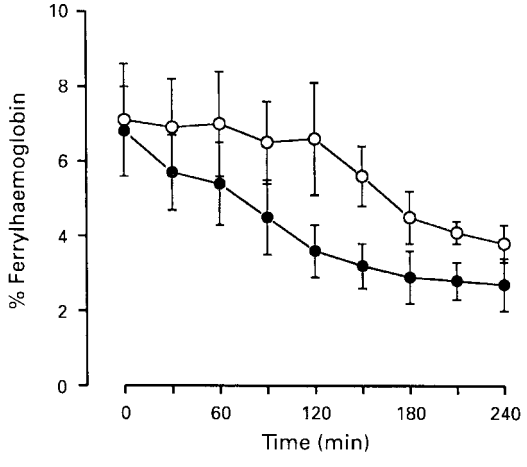

Figure 5 Changes in ferrylhaemoglobin concentrations during ABAP-induced haemolysis (expressed as percentage total haem; mean, 95 % confidence intervals, $n = 6$ in each case). The concentrations of ferrylhaemoglobin in flasks containing propofol 50 μmol litre^{-1} (●) and an equivalent quantity of Intralipid (○) are shown. There was no difference in the concentrations of ferrylhaemoglobin between Intralipid- and saline-containing flasks, and the effect of intermediate concentrations of propofol (12.5 and 25 μmol litre^{-1}) was between the effects of propofol 50 μmol litre^{-1} and Intralipid (data not shown for the sake of clarity).

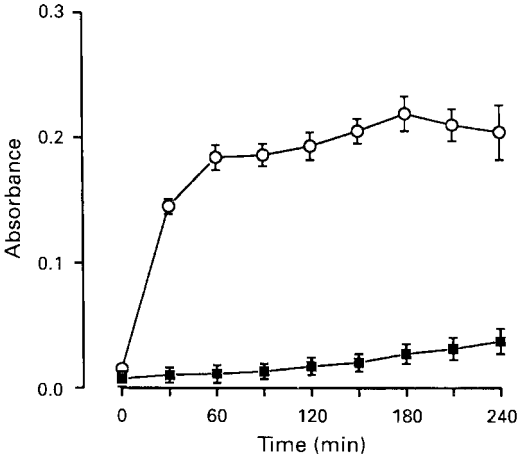

Figure 6 Absorption at 620 nm during ABAP-induced haemolysis in the presence of Na_2S 8 mmol litre^{-1} (mean, 95 % confidence intervals) ○ = Saline, ■ = non-haemolysing control ($n = 6$).

haemoglobin and sulphide) increased rapidly in the red cell suspensions to which ABAP had been added, but only very slowly in the blank controls (fig. 6). Thus the formation of ferrylhaemoglobin during the early stages of the process was confirmed.

HAEMOGLOBIN OXIDATION EXPERIMENTS

The results of these experiments are shown in table 2. Oxidation of methaemoglobin with a 20-fold excess of H_2O_2 generated significant quantities of ferrylhaemoglobin, with a corresponding reduction in the proportion of methaemoglobin. Serial spectroscopic analysis over the subsequent 30 min demonstrated a gradual reduction in the concentration of the ferryl species accompanied by a corresponding increase in methaemoglobin concentrations. This transition was accelerated by propofol, but not by saline, Intralipid or thiopentone.

Discussion

Several workers have used models of oxidant-mediated haemolysis to investigate the susceptibility of intact cell membranes to oxidant stress and the efficacy of potential antioxidants, although the relationship between this and antioxidant activity in other tissues has not been well defined. Stocks and Dormandy, while commenting on the poor reproducibility of the assay, have shown that H_2O_2-induced haemolysis is inhibited by butylated hydroxyanisole and α-tocopherol [2], both established antioxidants. Haemolysis induced by azo compounds is much more predictable [4–6]. Using this model, Miki and co-workers have demonstrated in rats that dietary-induced deficiency of α-tocopherol enhances susceptibility to haemolysis induced by the water-soluble azo compound ABAP [6] and that antioxidants such as uric acid, ascorbic acid, cysteine, glutathione and water-soluble analogues of vitamin E confer protection *in vitro* [4]. The biochemical mechanisms underlying ABAP-induced lysis of erythrocytes are not defined, although recent studies suggest that radical-mediated injury to membrane lipids is not an important process in this model, and that damage to non-lipid membrane elements such as ion transport proteins is more likely to be responsible [17].

Table 2 Methaemoglobin oxidation experiments. Concentrations of met- and ferrylhaemoglobin (Hb) (expressed as percentage of total haem, mean (SEM) before and after oxidatin of methaemoglobin with H_2O_2, and then 0, 10, 20 and 30 min after the subsequent addition of saline, Intralipid, thiopentone 250 μmol litre^{-1} or propofol 50 μmol litre^{-1}. Concentrations were analysed at each stage by one-way analysis of variance (ANOVA), and groups were compared with the propofol group using a *t* test to which a Bonferroni correction was applied (⋆$P < 0.05$, ⋆⋆$P < 0.01$). †Little ferrylhaemoglobin would be expected in the native preparation. These low values probably represent the inaccuracy in measuring the absorbance at the particular wavelengths used

	Saline	Intralipid	Thiopentone	Propofol	*P* (ANOVA)
%MetHb					
Hb alone	83.9 (1.5)	83.3 (1.6)	83.8 (1.5)	84.0 (1.5)	ns
$+H_2O_2$	24.3 (1.9)	23.1 (1.5)	21.6 (1.5)	22.8 (1.3)	ns
0 min	35.1 (2.1)	35.8 (2.6)	35.3 (2.3)	41.8 (2.5)	ns
10 min	45.0 (2.5)⋆	45.9 (2.6)⋆	46.4 (2.3)⋆	58.4 (2.6)	0.003
20 min	50.2 (2.3)⋆⋆	52.3 (2.3)⋆	52.5 (2.1)⋆	64.0 (2.1)	0.0012
30 min	54.4 (2.3)⋆⋆	55.2 (2.1)⋆⋆	55.8 (2.0)⋆	67.8 (1.9)	0.0004
%FerrylHB					
Hb alone†	< 6	< 6	< 6	< 6	ns
$+H_2O_2$	31.5 (0.6)	32.1 (0.5)	32.7 (0.6)	32.8 (0.5)	ns
0 min	27.0 (0.9)	27.4 (0.9)	27.4 (0.8)	24.9 (0.9)	ns
10 min	23.6 (0.9)⋆	23.5 (0.9)⋆	23.1 (0.8)⋆	18.3 (1.0)	0.0011
20 min	21.7 (0.8)⋆	20.9 (0.8)⋆	20.7 (0.8)⋆	16.2 (0.8)	0.0003
30 min	19.9 (0.9)⋆⋆	19.7 (0.7)⋆⋆	19.3 (0.7)⋆⋆	14.8 (0.7)	0.0002

In this study we have demonstrated that propofol, and to a lesser degree thiopentone, inhibited ABAP-induced haemolysis. Propofol (P-OH) is a phenol-based antioxidant and a potent inhibitor of lipid peroxidation *in vitro* [8, 9] and *in vivo* [18]. For example, it scavenges lipid peroxyl radicals ($LO_2\cdot$) through a process of hydrogen atom transfer to form a relatively stable propofol phenoxyl radical (P-O·) and a lipid hydroperoxide (LOOH) (reaction 5) [8]:

$$P\text{–}OH + LO_2\cdot \rightarrow P\text{–}O\cdot + LOOH \qquad (5)$$

It is not clear if inhibition of ABAP-induced haemolysis by propofol resulted from scavenging of organic peroxyl radicals generated by ABAP (reaction 3), scavenging of lipid peroxyl radicals subsequently generated by it, or interruption of some other oxidant-mediated process initiated within, or close to, the cell membrane (e.g. generation of ferrylhaemoglobin species which have been shown to induce lipid peroxidation). Thiopentone has also been shown to inhibit lipid peroxidation *in vitro*, although the mechanisms for such an activity have not been defined.

Difficulties in explaining the inhibitory effects of propofol and thiopentone in this model result in part from the lack of information on the mechanisms of ABAP-induced haemolysis. It has been hypothesized that the release of iron from iron-containing proteins in tissues exposed to an oxidative stress may lead to a second wave of radical-mediated injury as a result of the ability of the released iron to catalyse further radical generation via the Haber–Weiss cycle (reactions 6–9) [1]:

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2\cdot^- \qquad (6)$$

$$O_2\cdot^- + O_2\cdot^- + 2H^+ \rightarrow H_2O_2 + O_2 \qquad (7)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \cdot OH \qquad (8)$$

$$O_2\cdot^- + Fe^{3+} \rightarrow Fe^{2+} + O_2 \qquad (9)$$

It is possible therefore that the release of haemoglobin during ABAP-induced haemolysis may contribute to subsequent erythrocyte membrane damage. Previous workers have dismissed this hypothesis because they could find no evidence for a change in the redox state of haemoglobin during this process [6]. In contrast, this study has shown that considerable methaemoglobin was formed during this process, although the demonstration that the concentrations were highest in the propofol-containing flasks (that is, the flasks in which haemolysis was delayed) does not ostensibly support the proposition that haem-bound iron contributes to erythrocyte oxidative injury.

The capacity of haem-containing proteins such as haemoglobin and myoglobin to generate radicals is thought to result in part from the ability of haem-bound iron to undergo one-electron transfer (oxidation–reduction) reactions and thereby oscillate between redox states (reaction 1). The absorption spectra of these haemoglobin species are distinct, allowing their relative proportions to be measured in both cellular and non-cellular preparations [13, 14]. How such redox transitions account for radical generation is not however entirely clear. It may result from the formation of the highly reactive hydroxyl radical via the Haber–Weiss cycle (·OH, reactions 6–9), although several studies have demonstrated that such processes do not occur with intact haem proteins such as haemoglobin [1]. Other workers have suggested that haemoglobin and myoglobin may generate oxidant stress in tissues which is not mediated by the formation of ·OH [13, 19], but which results from radical formation within amino acid residues of the globin element of the molecule (which can be represented by $-XH\rightarrow -X\cdot$). Thus, the oxidation of methaemoglobin ($HX\text{-}HbFe^{3+}$) by H_2O_2 and other hydroperoxides produces, via a two-electron transfer reaction, ferrylhaemoglobin ($\cdot X\text{-}HbFe^{4+}\text{-}OH$) [20] which is an unstable and reactive species in which the haem iron is one oxidizing equivalent above the met-state, with the second oxidizing equivalent present as a radical

species on a protein residue within the globin moiety (as a tyrosine-derived phenoxyl radical):

$$HX\text{-}HbFe^{3+} + H_2O_2 \rightarrow \cdot X\text{-}HbFe^{4+}\text{-}OH + H_2O \quad (10)$$

Myoglobin behaves in a similar manner [21, 22] and in so doing promotes lipid peroxidation in cell membrane systems, although it is not clear if this is initiated by the protein radical or the ferrylhaem element of the molecule [12]. This injury can be attenuated by thiol-containing compounds such as *N*-acetylcysteine and *N*-(2-mercaptopropionyl)glycine [12]. Generation of ferrylhaemoglobin is also associated with increased concentrations of peroxidation in a ruptured erythrocyte preparation [23].

In order to explain the (higher) concentrations of methaemoglobin generated during ABAP-induced haemolysis in the presence of propofol we hypothesize that the organic hydroperoxide generated by ABAP (ROOH, reaction 4) oxidizes oxyhaemoglobin ($HX\text{-}HbFe^{2+}\text{-}O_2$) to the ferryl state ($\cdot X\text{-}HbFe^{4+}\text{-}OH$) :

$$ROOH + HX\text{-}HbFe^{2+}\text{-}O_2 \rightarrow ROH + \cdot X\text{-}HbFe^{4+}\text{-}OH + O_2 \quad (11)$$

in a manner analogous to the peroxidation by *hydrogen* peroxide (*H*OOH) described above (reaction 10). We suggest further that propofol (P–OH) reduces this ferryl species back to methaemoglobin thus:

$$P\text{–}OH + \cdot X\text{–}HbFe^{4+}\text{–}OH \rightarrow HX\text{–}HbFe^{4+} + \text{–}OH + P\text{–}O\cdot \quad (12)$$

$$P\text{–}OH + HX\text{–}HbFe^{4+}\text{–}OH \rightarrow HX\text{–}HbFe^{3+} + P\text{–}O\cdot + H_2O \quad (13)$$

The formation of ferrylhaemoglobin during ABAP-induced haemolysis and reduced concentrations of this species in the propofol-containing flasks supports the first part of this hypothesis. The second series of experiments provided more definitive evidence that propofol, but not thiopentone, Intralipid or saline, promoted the reduction of ferrylhaemoglobin to methaemoglobin (reactions 12 and 13). It is not clear if generation of ferrylhaemoglobin is directly responsible for the oxidative stress to which erythrocytes are exposed when incubated with ABAP, although the association between reduced concentrations of ferrylhaemoglobin and lower rates of haemolysis in the propofol-containing flasks would support this contention.

We conclude from this *in vitro* study that propofol is capable of protecting erythrocytes against oxidant stress. Although this effect has been demonstrated at concentrations relevant to anaesthesia (12.5–50 μmol litre^{-1} is equivalent to 2.2–8.8 μg ml^{-1}), further work is required to demonstrate that this is a clinically significant effect. The mechanisms whereby propofol protects against ABAP-induced haemolysis remain to be fully elucidated. In this respect, although propofol inhibits lipid peroxidation, we have shown in these experiments that there is a further aspect to the antioxidant properties of this agent. Thus propofol can interact with high oxidation-state species of haemoglobin, and hence reduce ferrylhaemoglobin to the met-state. The reduction in the concentrations of these damaging high-oxidation state species may contribute to the protection afforded to membrane structures by the presence of this lipid-soluble radical scavenging molecule.

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References

1. Halliwell B, Gutteridge JMC. Role of free radicals and catalytic metal ions in human disease: an overview. *Methods in Enzymology* 1990; **186**: 1–85.
2. Stocks J, Dormandy TL. The autoxidation of human red cell lipids induced by hydrogen peroxide. *British Journal of Haematology* 1971; **20**: 95–111.
3. Kellogg EW, Fridovich I. Liposome oxidation and erythrocyte lysis by enzymically generated superoxide and hydrogen peroxide. *Journal of Biological Chemistry* 1977; **252**: 6721–6728.
4. Niki E, Komuro E, Takahashi M, Urano S, Ito E, Terao K. Oxidative hemolysis of erythrocytes and its inhibition by free radical scavengers. *Journal of Biological Chemistry* 1988; **263**: 19809–19814.
5. Niki E. Free radical initiators as source of water- or lipid-soluble peroxyl radicals. *Methods in Enzymology* 1990; **186**: 100–108.
6. Miki M. Tamai H, Mino M, Yamamoto Y, Niki E. Free-radical chain oxidation of rat red blood cells by molecular oxygen and its inhibition by α-tocopherol. *Archives of Biochemistry and Biophysics* 1987; **258**: 373–380.
7. Giulivi C, Davies KJA. A novel antioxidant role for haemoglobin. The comproportionation of ferrylhaemoglobin with oxyhemoglobin. *Journal of Biological Chemistry* 1990; **265**: 19453–19460.
8. Murphy PG, Myers DS, Davies MJ, Webster NR, Jones JG. The antioxidant potential of propofol (2,6-diisopropylphenol). *British Journal of Anaesthesia* 1992; **68**: 613–618.
9. Musacchio E, Rizzoli V, Bianchi M, Bindoli A, Galzigna L. Antioxidant action of propofol on liver microsomes, mitochondria and brain synaptosomes in the rat. *Pharmacology and Toxicology* 1991; **69**: 75–77.
10. Aarts L, van der Hee R, Dekker I, de Jong J, Langemeijer H, Bast A. The widely used anesthetic agent propofol can replace α-tocopherol as an antioxidant. *FEBS Letters* 1995; **357**: 83–85.
11. Demopoulos HB, Flamm ES, Seligman ML, Jorgensen E, Ransohoff J. Antioxidant effects of barbiturates in model membranes undergoing free radical damage. *Acta Neurologica Scandinavica* 1977; **56**: 152–153.
12. Turner JJO, Rice-Evans CA, Davies MJ, Newman ESR. The formation of free radicals by cardiac myocytes under oxidative stress and the effects of electron-donating drugs. *Biochemical Journal* 1991; **277**: 833–837.
13. Winterbourn CC. Oxidative reactions of hemoglobin. *Methods in Enzymology* 1990; **186**: 265–273.
14. Berzofsky JA, Peisach J, Blumberg WE. Sulheme proteins. 1. Optical and magnetic properties of sulfmyoglobin and its derivatives. *Journal of Biological Chemistry* 1971; **246**: 3367–3377.
15. Armitage P, Berry G. *Statistical Methods in Medical Research*, 3rd Edn. Oxford: Blackwell Scientific Publications, 1994; 234.
16. Altman DG. *Practical Statistics in Medical Research*. London: Chapman and Hall, 1991; 212–213.
17. Sandhu IS, Ware K, Grisham MB. Peroxyl radical-mediated haemolysis: role of lipid, protein and sulfydryl oxidation. *Free Radical Research Communications* 1992; **16**: 111–122.
18. Murphy PG, Bennett JR, Myers DS, Davies MJ, Jones JG. The effect of propofol on free radical-induced lipid peroxidation in rat liver microsomes. *European Journal of Anaesthesiology* 1993; **10**: 261–266.

19. Puppo A, Halliwell B. Formation of hydroxyl radicals from hydrogen peroxide in the presence of iron. Is haemoglobin a biological Fenton reagent? *Biochemical Journal* 1988; **249**: 185–190.
20. McArthur KM, Davies MJ. Detection and reactions of the globin radical in haemoglobin. *Biochimica Biophysica Acta* 1993; **1202**: 173–181.
21. Davies MJ. Detection of myoglobin-derived radicals on reaction of metmyoglobin with hydrogen peroxide and other peroxidic compounds. *Free Radical Research Communications* 1990; **10**: 361–370.
22. Whitburn KD, Shieh JJ, Sellers RM, Hoffman MZ, Taub IA. Redox transformations in ferrimyoglobin induced by radiation-generated free radicals in aqueous solution. *Journal of Biological Chemistry* 1992; **257**: 1860–1869.
23. Paganga G, Rice-Evans C, Rule R, Leake D. The interaction between ruptured erythrocyte and low-density lipoproteins. *FEBS Letters* 1992; **303**: 154–158.