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Lipid peroxidation and haemoglobin degradation in red blood cells exposed to t-butyl hydroperoxide

The relative roles of haem- and glutathione-dependent decomposition of t-butyl hydroperoxide and membrane lipid hydroperoxides in lipid peroxidation and haemolysis

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Red cells exposed to t-butyl hydroperoxide undergo lipid peroxidation, haemoglobin degradation and hexose monophosphate-shunt stimulation. By using the lipid-soluble antioxidant 2,6-di-t-butyl-*p*-cresol, the relative contributions of t-butyl hydroperoxide and membrane lipid hydroperoxides to oxidative haemoglobin changes and hexose monophosphate-shunt stimulation were determined. About 90% of the haemoglobin changes and all of the hexose monophosphate-shunt stimulation were caused by t-butyl hydroperoxide. The remainder of the haemoglobin changes appeared to be due to reactions between haemoglobin and lipid hydroperoxides generated during membrane peroxidation. After exposure of red cells to t-butyl hydroperoxide, no lipid hydroperoxides were detected iodimetrically, whether or not glucose was present in the incubation. Concentrations of 2,6-di-t-butyl-*p*-cresol, which almost totally suppressed lipid peroxidation, significantly inhibited haemoglobin binding to the membrane but had no significant effect on hexose monophosphate shunt stimulation, suggesting that lipid hydroperoxides had been decomposed by a reaction with haem or haem-protein and not enzymically via glutathione peroxidase. The mechanisms of lipid peroxidation and haemoglobin oxidation and the protective role of glucose were also investigated. In time-course studies of red cells containing oxyhaemoglobin, methaemoglobin or carbonmono-oxyhaemoglobin incubated without glucose and exposed to t-butyl hydroperoxide, haemoglobin oxidation paralleled both lipid peroxidation and t-butyl hydroperoxide consumption. Lipid peroxidation ceased when all t-butyl hydroperoxide was consumed, indicating that it was not autocatalytic and was driven by initiation events followed by rapid propagation and termination of chain reactions and rapid non-enzymic decomposition of lipid hydroperoxides. Carbonmono-oxyhaemoglobin and oxyhaemoglobin were good promoters of peroxidation, whereas methaemoglobin relatively spared the membrane from peroxidation. The protective influence of glucose metabolism on the time course of t-butyl hydroperoxide-induced changes was greatest in carbonmono-oxyhaemoglobin-containing red cells followed in order by oxyhaemoglobin- and methaemoglobin-containing red cells. This is the reverse order of the reactivity of the hydroperoxide with haemoglobin, which is greatest with methaemoglobin. In studies exposing red cells to a wide range of t-butyl hydroperoxide concentrations, haemoglobin oxidation and lipid peroxidation did not occur until the cellular glutathione had been oxidized. The amount of lipid peroxidation per increment in added t-butyl hydroperoxide was greatest in red cells containing carbonmono-oxyhaemoglobin, followed in order by oxyhaemoglobin and methaemoglobin. Red cells containing oxyhaemoglobin and carbonmono-oxyhaemoglobin and exposed to increasing concentrations of t-butyl hydroperoxide became increasingly resistant to lipid peroxidation as methaemoglobin accumulated, supporting a relatively protective role for methaemoglobin. In the presence of glucose, higher levels of t-butyl hydroperoxide were required to induce lipid peroxidation and haemoglobin oxidation compared with

incubations without glucose. Carbonmono-oxyhaemoglobin-containing red cells exposed to the highest levels of t-butyl hydroperoxide underwent haemolysis after a critical level of lipid peroxidation was reached. Inhibition of lipid peroxidation by 2,6-di-t-butyl-*p*-cresol below this critical level prevented haemolysis. Oxidative membrane damage appeared to be a more important determinant of haemolysis *in vitro* than haemoglobin degradation. The effects of various antioxidants and free-radical scavengers on lipid peroxidation in red cells or in ghosts plus methaemoglobin exposed to t-butyl hydroperoxide suggested that red-cell haemoglobin decomposed the hydroperoxide by a homolytic scission mechanism to t-butoxyl radicals.

In recent years there has been extensive research on lipid peroxidation and the effects of organic hydroperoxides on biological systems. The process of lipid peroxidation has been subdivided into three phases: initiation, propagation and termination (Witting, 1980). The ability of the red cell to limit the extent of propagation, hasten termination and prevent the destruction of biomembrane architecture caused by lipid peroxidation chain reactions may be linked to its capacity to inactivate lipid hydroperoxides.

We have studied red cells exposed to t-butyl hydroperoxide as a model for the effects of lipid peroxidation in a metabolically active cell. Depending on the availability of glucose and the liganded state of haemoglobin, lipid peroxidation and haemoglobin alterations represent extremes of a spectrum of oxidative damage (Trotta *et al.*, 1981). The extent of lipid peroxidation depended on the balance between the presence of initiators of lipid peroxidation (HbO_2 and low concentrations of metHb) and terminators of lipid peroxidation (GSH, ascorbate, high concentrations of metHb) (Trotta *et al.*, 1982). These studies demonstrated a critical role for the HMS as mediated by GSH, ascorbate and GSH peroxidase.

The present study was designed to assess further the protective role of glucose metabolism and determine the mechanism of t-butyl hydroperoxide-induced haemoglobin oxidation and lipid peroxidation. We studied the effects of various levels of t-butyl hydroperoxide in red cells to determine the capacity of glucose metabolism (via GSH peroxidase) to effect reduction of hydroperoxides and to determine the nature of the reactions between unmetabolized hydroperoxides and other cellular constituents, particularly haemoglobin. We have used the lipid soluble antioxidant BHT to suppress membrane peroxidation induced by t-butyl hydroperoxide and prevent formation of lipid hydroperoxides. We have been able to separate the relative roles of t-butyl hydroperoxide and lipid hydroperoxides in causing haemoglobin changes and HMS stimulation via GSH peroxidase.

The present study was also aimed at determining differences between the lipid peroxidation process in purified lipid/catalyst systems (Tappel, 1953) and biological systems. The time course of oxidative alterations and hydroperoxide consumption were investigated in red cells and their isolated ghosts exposed to t-butyl hydroperoxide to determine the relative roles of initiation, propagation and termination reactions in the peroxidation of the intact red-cell membrane.

Abbreviations used: BHT, butylated hydroxytoluene (2,6-di-t-butyl-*p*-cresol); DETAPAC, diethylenetriamine-penta-acetic acid; DPA, diphenylamine; DPF, diphenylfuran; GSH, reduced glutathione; HbO_2, oxyhaemoglobin; metHb, methaemoglobin; HbCO, carbonmono-oxyhaemoglobin; intact Hb, intact haemoglobin (defined as the sum of HbO_2 plus metHb); HMS, hexose monophosphate shunt; KRP buffer, Krebs–Ringer phosphate buffer; PBS, phosphate-buffered saline; TBAR, thiobarbituric acid-reactive products of lipid peroxidation; t-Bu, t-butyl.

Materials and methods

Red-cell preparations

Adult human blood was drawn daily into a test tube containing a small amount of 3.8% sodium citrate solution. After centrifugation plasma and white cells were removed and red cells were washed three times with PBS (nine parts 0.9% NaCl, one part 0.1 M-KH_2PO_4/K_2HPO_4, pH 7.4). Experiments were carried out with three types of red-cell preparations, characterized by the haemoglobin type (HbO_2, metHb or HbCO). Red cells containing HbO_2 were taken directly from the washed red-cell pellets. Red cells containing metHb were prepared by incubating a volume of packed red cells with an equal volume of 0.5% $NaNO_2$ in half-concentrated PBS for 10 min at 25°C. After incubation red cells were washed five times with PBS to remove nitrite. Stock red-cell suspensions containing HbCO were prepared by blowing CO over a 20% (v/v) suspension of red cells in PBS until the visible spectra of red-cell lysates reached a maximum at 569 nm. All subsequent incubations of red cells containing HbCO were carried out in air-tight stoppered flasks under an atmosphere of air/CO (9:1).

Incubations with t-butyl hydroperoxide

Incubations were carried out in 25 ml flasks containing 0.25 ml of 2 mM-t-butyl hydroperoxide

(Sigma) in PBS and 0.25ml of 20% (v/v) red-cell suspension in PBS brought to a final volume of 5ml with KRP buffer (120mM-NaCl/4.8mM-KCl/1.3mM-$CaCl_2$/1.2mM-$MgSO_4$/16.5mM-NaH_2PO_4/Na_2HPO_4, pH7.4). The final concentrations were 0.1mM-t-butyl hydroperoxide and 1% (v/v) red cells. Variable additions included (at final concentrations) 3.75mM-glucose and 5 or 25μM-BHT as 10μl of a concentrated solution in propan-2-ol (10μl of propan-2-ol was added to incubations without BHT). After completing all additions, the 25ml reaction flasks were closed with rubber stoppers and were incubated for 1h at 37°C in a shaking water bath. Studies of the time course of t-butyl hydroperoxide-induced lipid peroxidation and haemoglobin alterations and the effect of antioxidants and free-radical scavengers on incubations of red cells were carried out at 37°C for 1h in 10ml flasks containing 0.1ml of 2mM-t-butyl hydroperoxide (Sigma) in PBS and 0.1ml of 20% (v/v) red-cell suspension in PBS brought to a final volume of 2ml with KRP buffer. Some incubations included 3.75mM-glucose. In studies using antioxidants and free-radical scavengers, all compounds were added as 20μl portions of concentrated stock solutions. Stock solutions of GSH (Sigma), thiourea (Fisher Scientific Co.), DETAPAC (Sigma) and histidine monohydrochloride monophosphate (Sigma) adjusted to pH7.4 with NaOH, were prepared in water. Monosodium urate (Sigma) was prepared in 0.05M-NaOH. Sodium ascorbate (Sigma) was prepared in 1mM-KCl, pH2.0. Stock solutions of BHT (Aldrich Chemical Co.), DPF (Eastman Kodak Co.) and DPA (Eastman Chemical Co.) were prepared in propan-2-ol. In studies of the effects of various concentrations of t-butyl hydroperoxide on red cells, incubations were carried out at 37°C for 90min in 25ml flasks containing 0.2ml of a stock solution of t-butyl hydroperoxide in PBS and 0.2ml of 20% (v/v) red cell suspension in PBS brought to a final volume of 2ml with KRP buffer. Incubations were stopped by centrifuging at 1200*g* for 5min. Supernatants were removed and used for measurement of TBAR and residual t-butyl hydroperoxide. Red-cell pellets were used for studies of haemoglobin. After some incubations, red-cell pellets were lysed in ice-cold 30mosM-NaH_2PO_4, pH7.4, centrifuged at 27000*g* for 10min at 4°C and the red-cell ghost lipids were analysed as described below.

Purification of methaemoglobin

Methaemoglobin was prepared from red cells as described previously (Trotta *et al.*, 1981).

Incubation of red-cell ghosts with metHb and t-butyl hydroperoxide

Red-cell ghosts were prepared from freshly drawn adult human blood by the method of Dodge *et al.* (1962) as modified by Burton *et al.* (1981). Ghosts obtained were virtually completely free of haemoglobin. Ghosts were not resealed. Incubation mixtures consisted of 0.75ml of 20% (v/v) red-cell equivalent ghost suspensions in 1.25mM-NaH_2PO_4/Na_2HPO_4, pH8.0, 0.15ml of stock metHb solution in 0.125M-KH_2PO_4/K_2HPO_4, pH7.4, and 2.1ml of KRP buffer. t-Butyl hydroperoxide was added as a small portion of 6mM-aqueous stock concentrate. Some experiments included antioxidants and free-radical scavengers, which were added as 20μl portions of concentrated stock solutions as described above for all red-cell incubations. Incubations were carried out at 37°C either in a shaking water bath or in the magnetically stirred compartment of a Yellow Springs oxygen electrode to measure O_2 consumption. TBAR were assayed as described below.

Measurement of lipid peroxidation

Lipid peroxidation was assessed by measuring TBAR and membrane lipid hydroperoxides. A portion (2ml) of the supernatant fraction from incubations with red cells was mixed with 1ml of 30% (w/v) trichloroacetic acid and centrifuged at 5000*g* for 7min; after incubation of non-cellular experiments 2vol. of the red-cell ghost/metHb/t-butyl hydroperoxide incubation mixture were mixed with 1vol. of 30% trichloroacetic acid and the resulting mixture was centrifuged at 5000*g* for 5min. The supernatants were assayed for TBAR as described previously (Trotta *et al.*, 1982). For measurement of lipid hydroperoxides 10ml incubation volumes were used to increase the yields of membrane lipids. After incubation with t-butyl hydroperoxide, red cells were centrifuged and the 100μl pellet was lysed by addition of 10ml of ice-cold 15mM-NaH_2PO_4, pH7.4, which sometimes also included 10mM-NaCN (brought to pH7.4 with NaOH) to prevent haem- and haem-protein-promoted decomposition of hydroperoxides during the extraction procedure (O'Brien, 1969). The lysate was centrifuged at 27000*g* for 10min at 4°C. To the membrane pellet was added 1.5ml of propan-2-ol with rapid mixing; this mixture was allowed to sit on ice for 20min. Then 10ml of chloroform was added and this was allowed to extract for 1h on ice. The extract was filtered through glass wool and the propan-2-ol/chloroform was evaporated off under a stream of ultrapure N_2 in an Organomation Evaporator maintained at 37°C. The residue was redissolved into 0.2ml of methanol for the hydroperoxide assay. Lipid hydroperoxides in the extracted red-cell-ghost lipids and residual t-butyl hydroperoxide in supernatants from red-cell incubations were assayed iodimetrically as described previously (Trotta *et al.*, 1982). The limit of detection of the assay was about 5μM-hydroperoxide in the injected 0.1ml sample (about 10–20nmol of hydroperoxide per ml of red cells).

Analysis of red cell haemoglobin and haemolysis

The amounts of HbO_2, metHb and non-intact Hb (defined as the total of all haemoglobin species other than HbO_2 and metHb) in red cells exposed to t-butyl hydroperoxide were determined as described previously (Trotta *et al.*, 1982). Haemolysis was assessed by the following procedure. A portion (0.1 ml) of incubation mixture was mixed with 1.9 ml of either PBS or water. The light scattering by these mixtures was measured at 740 nm in a Cary 14 spectrophotometer. Percentage haemolysis is calculated from the equation:

$$\text{Haemolysis (\%)} = 100 \times (A_{740}-B_{740})/(C_{740}-B_{740})$$

where A_{740} is the light scattering of the red-cell suspension diluted into PBS, B_{740} is the light scattering of the red-cell suspension-lysed in water and C_{740} is the light scattering of a control red-cell suspension (no t-butyl hydroperoxide added) diluted in PBS. For measurement of haemoglobin precipitation, membrane pellets obtained from lysates from the haemoglobin assay above were resuspended in 20 mM-NaH_2PO_4 to 1% (v/v) red-cell equivalent. The light scattering of a 20-fold dilution of this suspension was measured at 740 nm in a Cary 14 spectrophotometer with a 1 cm light path. The A_{740} values obtained were reported as light scattering of a 1% (v/v) red cell equivalent membrane suspension.

Measurement of flux of glucose through the HMS

Flux of glucose through the HMS was measured as described previously (Trotta *et al.*, 1982).

Results

Oxidative effects of t-butyl hydroperoxide

As shown previously (Trotta *et al.*, 1981, 1982), red cells exposed to t-butyl hydroperoxide undergo lipid peroxidation and haemoglobin degradation. In HbO_2-containing red blood cells (Tables 1 and 2)

Table 1. *Effects of t-butyl hydroperoxide (t-BuOOH) and BHT on HbO_2-containing red cells*

A 1% (v/v) suspension of red cells containing HbO_2 was exposed to 0.1 mM-t-butyl hydroperoxide for 1 h at 37°C. Where indicated, 3.75 mM-glucose and/or BHT were present in the incubation medium during exposure to t-butyl hydroperoxide. After incubation measurements were made of lipid peroxidation (estimated by assay of the TBAR) and flux of glucose through the HMS.

Additions to incubation	TBAR (nM)*	Inhibition (%)	Flux through the HMS† (μmol of CO_2/h per ml of red cells)
t-BuOOH	372 ± 53		
t-BuOOH + 5 μM-BHT	167 ± 49	55	
t-BuOOH + 25 μM-BHT	43 ± 10	88	
Glucose			0.42 ± 0.09
Glucose + t-BuOOH	405 ± 51		3.54 ± 0.16 (100)
Glucose + t-BuOOH + 5 μM-BHT	140 ± 47	65	3.67 ± 0.23 (104) NS‡
Glucose + t-BuOOH + 25 μM-BHT	84 ± 34	79	3.75 ± 0.24 (106) NS

* Data are means of three experiments ± S.E.M.

† Data are means of three experiments ± S.E.M. Data are given as percentages in parentheses.

‡ Data from incubations with t-butyl hydroperoxide and BHT are compared with incubations with t-butyl hydroperoxide without BHT using Student's paired *t*-test. NS, not significant.

Table 2. *Effects of BHT on haemoglobin changes in HbO_2-containing red cells exposed to t-butyl hydroperoxide*

A 1% (v/v) suspension of red cells containing HbO_2 was exposed to 0.1 mM-t-butyl hydroperoxide for 1 h at 37°C. Where indicated, 3.75 mM-glucose and/or BHT were included in the incubation. After exposure to t-butyl hydroperoxide (t-BuOOH) measurements were made of HbO_2, metHb, non-intact Hb and haemoglobin precipitation.

Additions to incubation	HbO_2 (%)*		MetHb (%)*		Non-intact Hb (%)*		Precipitated Hb† (A_{740})	
None	98.7 ± 0.3		1.5 ± 0.4				0.036 ± 0.006	
t-BuOOH	24.6 ± 1.7		54.6 ± 1.5		20.9 ± 0.7		0.504 ± 0.012	
t-BuOOH + 5 μM-BHT	27.2 ± 0.7	NS‡	53.3 ± 0.9	NS	19.6 ± 0.4	NS	0.466 ± 0.016	NS
t-BuOOH + 25 μM-BHT	28.4 ± 0.3	NS	52.5 ± 0.4	NS	19.3 ± 0.4	NS	0.424 ± 0.016	$P < 0.05$
Glucose	98.2 ± 0.1		1.8 ± 0.3				0.044 ± 0.004	
Glucose + t-BuOOH	59.7 ± 1.8		30.6 ± 1.0		9.5 ± 1.0		0.364 ± 0.024	
Glucose + t-BuOOH + 5 μM-BHT	61.6 ± 2.2	$P < 0.05$	29.1 ± 1.5	NS	9.3 ± 1.3	NS	0.346 ± 0.024	NS
Glucose + t-BuOOH + 25 μM-BHT	64.6 ± 2.2	$P < 0.01$	28.9 ± 1.5	$P < 0.01$	6.6 ± 1.5	$P < 0.05$	0.316 ± 0.012	NS

* Data are the means of four experiments ± S.E.M. Non-intact Hb is defined as the sum of all haemoglobin species other than HbO_2 and metHb.

† Data are means of three experiments ± S.E.M.

‡ Levels of HbO_2, metHb, non-intact Hb and precipitated Hb in incubations with t-butyl hydroperoxide and BHT were compared with incubations with t-butyl hydroperoxide without BHT by Student's *t*-test. NS, not significant.

Table 3. *Effects of t-butyl hydroperoxide and BHT on MetHb-containing red cells*

A 1% (v/v) suspension of red cells containing MetHb was exposed to 0.1 mM-t-butyl hydroperoxide for 1 h at 37°C. Where indicated, 3.75 mM-glucose and/or BHT were present in the incubation medium during exposure to t-butyl hydroperoxide. After incubation measurements were made of lipid peroxidation (estimated by assay of the TBAR) and flux of glucose through the HMS.

Additions to incubation	TBAR (nM)*	Inhibition (%)	Flux through the HMS† (μmol of CO_2/h per ml of red cells)	
t-BuOOH	167 ± 34			
t-BuOOH + 5 μM-BHT	72 ± 29	57		
t-BuOOH + 25 μM-BHT	16 ± 16	90		
Glucose			0.50 ± 0.14	
Glucose + t-BuOOH	195 ± 25		1.12 ± 0.12 (100)	
Glucose + t-BuOOH + 5 μM-BHT	68 ± 7	65	1.08 ± 0.13 (96)	NS‡
Glucose + t-BuOOH + 25 μM-BHT	43 ± 19	78	1.06 ± 0.12 (95)	$P < 0.01$

* Data are means of three experiments ± S.E.M.

† Data are means of three experiments ± S.E.M. Data are given as percentages in parentheses.

‡ Data from incubations with t-butyl hydroperoxide and BHT are compared with incubations with t-butyl hydroperoxide without BHT using Student's paired *t*-test. NS, not significant.

Table 4. *Effects of BHT on haemoglobin changes in metHb-containing red cells exposed to t-butyl hydroperoxide*

A 1% (v/v) suspension of red cells containing metHb was exposed to 0.1 mM-t-butyl hydroperoxide for 1 h at 37°C. Where indicated, 3.75 mM-glucose and/or BHT were included in the incubation. After exposure to t-butyl hydroperoxide (t-BuOOH) measurements were made of HbO_2, metHb, non-intact Hb and haemoglobin precipitation.

Additions to incubation	HbO_2 (%)*		MetHb (%)*		Non-intact Hb (%)*		Precipitated Hb† (A_{740})	
None	3.2 ± 0.2		96.8 ± 0.2				0.066 ± 0.004	
t-BuOOH	0.6 ± 0.4		57.5 ± 1.7		41.9 ± 1.4		1.246 ± 0.044	
t-BuOOH + 5 μM-BHT	0.4 ± 0.3	NS‡	59.7 ± 1.6	$P < 0.01$	39.9 ± 1.6	$P < 0.01$	1.166 ± 0.018	NS
t-BuOOH + 25 μM-BHT	0.5 ± 0.3	NS	60.7 ± 1.6	$P < 0.05$	38.8 ± 1.5	$P < 0.05$	1.102 ± 0.004	$P < 0.05$
Glucose	16.1 ± 0.9		83.9 ± 0.9				0.066 ± 0.006	
Glucose + t-BuOOH	6.0 ± 1.0		60.1 ± 1.7		34.0 ± 1.3		1.084 ± 0.026	
Glucose + t-BuOOH + 5 μM-BHT	7.7 ± 1.1	$P < 0.05$	60.9 ± 1.4	NS	31.4 ± 1.1	$P < 0.01$	1.016 ± 0.040	NS
Glucose + t-BuOOH + 25 μM-BHT	9.3 ± 0.8	$P < 0.001$	60.5 ± 1.3	NS	30.5 ± 0.6	NS	0.966 ± 0.013	$P < 0.05$

* Data are the means of four experiments ± S.E.M.

† Data are means of three experiments ± S.E.M.

‡ Levels of HbO_2, metHb, non-intact Hb and precipitated Hb in incubations with t-butyl hydroperoxide and BHT were compared with incubations with t-butyl hydroperoxide without BHT by Student's *t*-test. NS, not significant.

Table 5. *Effects of t-butyl hydroperoxide and BHT on HbCO-containing red cells*

A 1% (v/v) suspension of red cells containing HbCO was exposed to 0.1 mM-t-butyl hydroperoxide for 1 h at 37°C. Where indicated, 3.75 mM-glucose and/or BHT were present in the incubation medium during exposure to t-butyl hydroperoxide. After incubation measurements were made of lipid peroxidation (estimated by assay of the TBAR) and flux of glucose through the HMS.

Additions to incubation	TBAR (nM)*	Inhibition (%)	Flux through the HMS† (μmol of CO_2/h per ml of red cells)	
t-BuOOH	829 ± 28			
t-BuOOH + 5 μM-BHT	689 ± 11	17		
t-BuOOH + 25 μM-BHT	279 ± 64	66		
Glucose			0.32 ± 0.05	
Glucose + t-BuOOH	444 ± 47		4.11 ± 0.36 (100)	
Glucose + t-BuOOH + 5 μM-BHT	315 ± 11	29	4.09 ± 0.31 (100)	NS‡
Glucose + t-BuOOH + 25 μM-BHT	112 ± 49	75	4.21 ± 0.28 (100)	NS

* Data are means of three experiments ± S.E.M.

† Data are means of three experiments ± S.E.M. Data are given as percentages in parentheses.

‡ Data from incubations with t-butyl hydroperoxide and BHT are compared with incubations with t-butyl hydroperoxide without BHT using Student's paired *t*-test. NS, not significant.

Table 6. *Effects of BHT on haemoglobin changes in HbCO-containing red cells exposed to t-butyl hydroperoxide*
A 1% (v/v) suspension of red cells containing HbCO was exposed to 0.1 mM-t-butyl hydroperoxide for 1 h at 37°C. Where indicated, 3.75 mM-glucose and/or BHT were included in the incubation. After exposure to t-butyl hydroperoxide (t-BuOOH) measurements were made of HbO_2, metHb, non-intact Hb and haemoglobin precipitation.

Additions to incubation	HbO_2 (%)*		MetHb (%)*		Non-intact Hb (%)*		Precipitated Hb† (A_{740})	
None	97.4 ± 0.4		2.6 ± 1.4				0.036 ± 0.006	
t-BuOOH	55.2 ± 1.3		29.2 ± 1.5		15.5 ± 1.7		0.240 ± 0.016	
t-BuOOH + 5 μM-BHT	59.3 ± 1.3	NS‡	28.4 ± 0.6	NS	12.4 ± 1.6	$P<0.05$	0.244 ± 0.020	NS
t-BuOOH + 25 μM-BHT	62.8 ± 0.6	$P<0.01$	26.5 ± 0.9	$P<0.05$	11.0 ± 0.8	$P<0.05$	0.184 ± 0.006	$P<0.05$
Glucose	97.0 ± 0.2		3.0 ± 0.2				0.036 ± 0.004	
Glucose + t-BuOOH	91.9 ± 1.8		5.5 ± 0.5		2.6 ± 1.4		0.064 ± 0.008	
Glucose + t-BuOOH + 5 μM-BHT	94.5 ± 0.2	NS	5.5 ± 0.2	NS	0	NS	0.050 ± 0	NS
Glucose + t-BuOOH + 25 μM-BHT	93.9 ± 0.5	NS	5.2 ± 0.1	NS	0.9 ± 0.4	NS	0.054 ± 0.004	NS

* Data are means of four experiments ± S.E.M. In the case of lysates prepared from red cells containing HbCO, haemoglobin was analysed by first incubating at 25°C with gentle shaking for 1 h under air until the visible spectra reached a maximum at 577 nm showing restoration of HbO_2 (see Trotta *et al.*, 1982).

† Data are means of three experiments ± S.E.M.

‡ Levels of HbO_2, metHb, non-intact Hb and precipitated Hb in incubations with t-butyl hydroperoxide and BHT were compared with incubations with t-butyl hydroperoxide but without BHT by Student's *t*-test. NS, not significant.

t-butyl hydroperoxide-induced lipid peroxidation was increased by the presence of glucose, whereas formation of metHb and non-intact Hb was decreased. t-Butyl hydroperoxide-induced Hb precipitation and binding to the membrane was inhibited by glucose metabolism. t-Butyl hydroperoxide caused a 9-fold increase in flux through the HMS. As observed previously, metHb-containing red cells (Tables 3 and 4) were resistant to t-butyl hydroperoxide-induced lipid peroxidation relative to HbO_2 and HbCO-containing red cells. Whereas glucose metabolism had no significant effect on lipid peroxidation, it significantly protected against non-intact Hb formation ($P<0.001$; four paired experiments) and haemoglobin precipitation ($P<0.05$; three paired experiments). t-Butyl hydroperoxide caused only a modest increase (140%) in flux through the HMS in metHb-containing red cells compared with that seen in HbO_2-containing red cells (800%). As observed previously, HbCO-containing red cells exposed to t-butyl hydroperoxide sustained the highest levels of lipid peroxidation relative to HbO_2- and metHb-containing red cells (Tables 5 and 6). HbCO-containing red cells were resistant to t-butyl hydroperoxide-induced non-intact Hb formation and haemoglobin precipitation when compared with HbO_2- and metHb-containing red cells. Glucose metabolism inhibited lipid peroxidation by 40% and almost completely protected against haemoglobin changes. As with HbO_2-containing red cells, t-butyl hydroperoxide stimulated HMS activity 14-fold over the controls.

Effects of BHT on t-butyl hydroperoxide-induced alterations in HbO_2-containing red cells

Tables 1 and 2 report the effects of BHT, a lipid-soluble antioxidant, on HbO_2-containing red cells exposed to t-butyl hydroperoxide. BHT inhibited TBAR formation in a dose-dependent manner; 25 μM-BHT almost completely inhibited lipid peroxidation but caused no significant change in HMS activity induced by t-butyl hydroperoxide. BHT had a small protective effect on t-butyl hydroperoxide-induced haemoglobin alterations. BHT (25 μM) inhibition of metHb and non-intact Hb formation was statistically significant in incubations including glucose. BHT (25 μM) also inhibited haemoglobin precipitation by 16% in incubations without glucose and 13% in incubations with glucose.

Effects of BHT on t-butyl hydroperoxide-induced alterations in metHb-containing red cells

As seen with HbO_2-containing red cells, BHT (25 μM) almost completely abolished lipid peroxidation in metHb-containing red cells (Tables 3 and 4). BHT caused a small (about 5%) inhibition of t-butyl hydroperoxide-induced flux through the HMS. BHT had a small but significant inhibitory effect on formation of non-intact Hb and haemoglobin precipitation in incubations without glucose. When glucose was included in the incubation, red cells incubated with t-butyl hydroperoxide plus BHT contained increased levels of HbO_2 and decreased formation of non-intact Hb and precipitated haemoglobin as compared with red cells incubated with t-butyl hydroperoxide and without BHT.

Effects of BHT on t-butyl hydroperoxide-induced alterations in HbCO-containing red cells

In HbCO-containing red cells exposed to t-butyl hydroperoxide (Tables 5 and 6), BHT strongly inhibited lipid peroxidation but had virtually no effect on flux through the HMS. Glucose metabolism almost completely protected against t-butyl hydro-

peroxide-induced haemoglobin alterations and obviated any protective effects of BHT. However, in incubations without glucose, BHT significantly protected against formation of metHb and inhibited non-intact Hb formation by 29% and haemoglobin precipitation by 23%.

HbCO-containing red cells were used to further characterize oxidation of membrane lipids induced by t-butyl hydroperoxide. Measurement of hydroperoxide levels iodimetrically in membrane lipids showed levels in the range of detection limits of the assay (10–20 nmol of hydroperoxide per ml of red cells). This extremely low level was seen whether or not glucose was included in the incubation.

The time course of t-butyl hydroperoxide-induced alterations in red cells

Fig. 1 shows the results of the time course of lipid peroxidation, haemoglobin alterations and t-butyl hydroperoxide consumption in red cells exposed to t-butyl hydroperoxide. The extent of lipid peroxidation is determined by the red cell haemoglobin such that HbCO > HbO_2 > metHb. In incubations without glucose and hence no HMS activity, the rate of t-butyl hydroperoxide consumption follows the order: metHb > HbO_2 > HbCO. In incubations without glucose the time course of haemoglobin oxidation parallels that of both lipid peroxidation and consumption of t-butyl hydroperoxide. For example, in HbO_2-containing red cells (Fig. 1*a*) exposed to t-butyl hydroperoxide and incubated without glucose the linear rate of lipid peroxidation is paralleled by linear rates of metHb and non-intact Hb formation and t-butyl hydroperoxide consumption. At 40 min 95% of the t-butyl hydroperoxide is consumed and lipid peroxidation and metHb and non-intact Hb formation cease. Similar results are seen with metHb- and HbCO-containing red cells (Figs. 1*b* and 1*c*).

The influence of glucose metabolism on the time course of t-butyl hydroperoxide-induced changes relates to the reactivity of the hydroperoxide with haemoglobin (Trotta *et al.*, 1981, 1982). In metHb-containing red cells (Fig. 1*b*), where metHb effects rapid reduction of t-butyl hydroperoxide and the lowest levels of lipid peroxidation, glucose metabolism had no significant effect on t-butyl hydroperoxide consumption, lipid peroxidation and haemoglobin changes. In HbO_2-containing red cells (Fig. 1*a*) the metHb reductase activity of glucose metabolism inhibits the rate of accumulation of metHb and non-intact Hb, resulting in decreased lipid peroxidation by 20 min (decreased availability of haem as initiator) and increased lipid peroxidation by 80 min (decreased availability of haem as terminator). In HbCO-containing red cells (Fig. 1*c*), where HbCO reacts more slowly with t-butyl hydroperoxide than does metHb, glucose metabolism effectively competes with HbCO for t-butyl hydroperoxide and significantly inhibits lipid peroxidation and formation of metHb and non-intact Hb when compared with incubations of HbCO-containing red cells without glucose. By 90 min, however, enough metHb and non-intact Hb has accumulated to allow increased haem-dependent initiation of lipid peroxidation. The levels of metHb and non-intact Hb are apparently not high enough to result in efficient haem-dependent termination and as a result experiments with HbCO-containing red cells incubated in the presence of glucose are the cases in which we observe propagation of lipid peroxidation after t-butyl hydroperoxide is consumed (90–120 min).

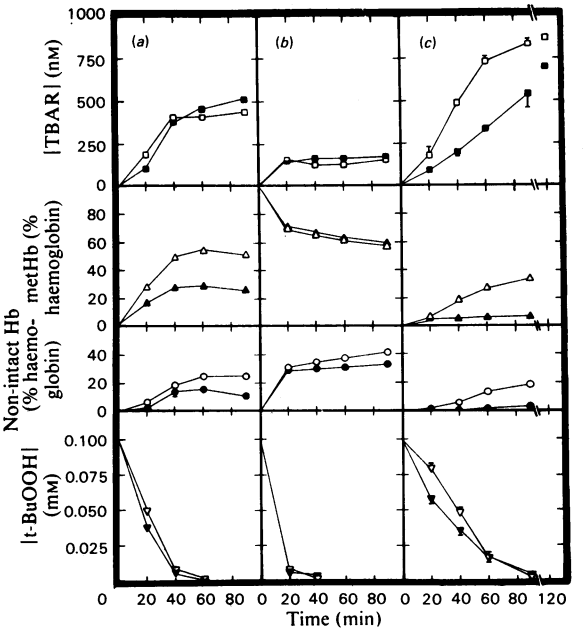

Fig. 1. *Time course of lipid peroxidation, haemoglobin changes and t-butyl hydroperoxide consumption by red cells exposed to t-butyl hydroperoxide*

A 1% (v/v) suspension of red cells containing HbO_2 (*a*), metHb (*b*) or HbCO (*c*) were incubated with (filled symbols) or without (open symbols) 3.75 mM-glucose and exposed to 0.1 mM-t-butyl hydroperoxide at 37°C. At certain times, incubation mixtures were rapidly centrifuged and supernatants were used to determine lipid peroxidation (estimated by the assay of TBAR) (□ and ■) and residual t-butyl hydroperoxide (t-BuOOH) (▽ and ▼), and red cell pellets were assayed for metHb (△ and ▲) and non-intact Hb (○ and ●) formation as described in the Materials and methods section. Each point represents the mean ± S.E.M. of three experiments (an S.E.M. bar is shown if it is larger than the point as drawn).

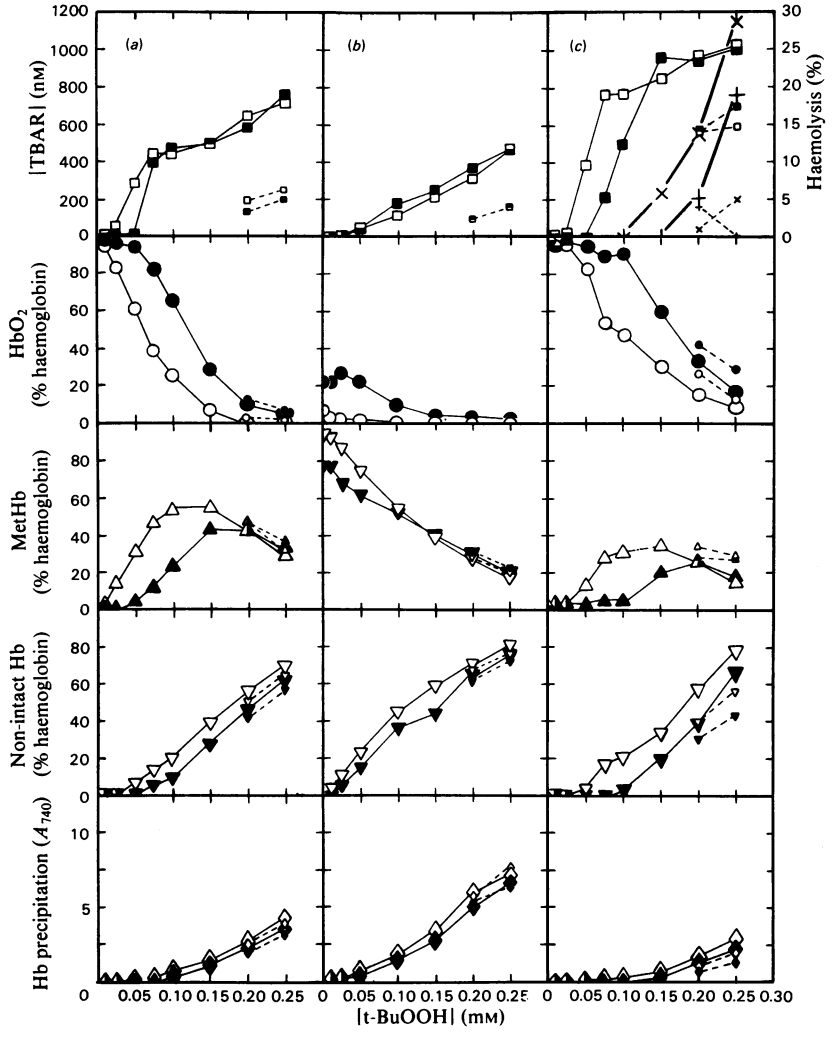

Fig. 2. *Lipid peroxidation, haemoglobin oxidation and precipitation and haemolysis in red cells exposed to various concentrations of t-butyl hydroperoxide*

A 1% (v/v) suspension of red cells containing HbO_2 (*a*), metHb (*b*) or HbCO (*c*) was incubated with (closed symbols) or without (open symbols) 3.75 mM-glucose and exposed to t-butyl hydroperoxide concentrations ranging from 0.010 to 0.250 mM for 90 min at 37°C. After exposure to t-butyl hydroperoxide measurements were made of lipid peroxidation (estimated by the assay of TBAR), levels of HbO_2, metHb and non-intact Hb, Hb precipitation and haemolysis (×, without glucose; +, with glucose). There was no haemolysis observed in incubations of red cells containing HbO_2 or metHb. At the highest concentrations of added t-butyl hydroperoxide (0.200 and 0.250 mM) 25 μM-BHT was included in the incubation as indicated by the small symbols connected by a broken line. Each point represents the mean of two experiments.

Oxidative alterations in red cells exposed to various concentrations of t-butyl hydroperoxide

In incubations of HbO_2- (Fig. 2*a*) and metHb-containing red cells (Fig. 2*b*) without glucose, no lipid peroxidation or haemoglobin oxidation is detected until added t-butyl hydroperoxide exceeds approx. 0.01 mM, which corresponds to the titration of the cellular GSH pool. Other investigators have shown that when HbO_2-containing red cells are exposed to H_2O_2 (Cohen & Hochstein, 1963) or t-butyl hydroperoxide (Ataullakhanov *et al.*, 1981), metHb formation does not occur until all of the intracellular GSH has been oxidized. In HbCO-containing red cells (Fig. 2*c*) incubated without glucose, added t-butyl hydroperoxide must exceed approx. 0.025 mM before detectable lipid peroxidation and haemoglobin oxidation occur, indicating an additional source of reducing equivalents. The

amount of lipid peroxidation per increment in t-butyl hydroperoxide is greatest in HbCO- and HbO_2-containing red cells and much lower in metHb-containing red cells. As soon as there is significant conversion of HbO_2 or HbCO into metHb and non-intact Hb (about 50%) the amount of peroxidation per t-butyl hydroperoxide concentration increment drops to that seen in metHb-containing red cells. Once metHb accumulates we see formation of non-intact Hb and haemoglobin precipitates. A small portion of membrane-bound haemoglobin may react with the peroxidizing lipids, as evidenced by inhibition of non-intact Hb formation and haemoglobin precipitation in red cells incubated with high concentrations of t-butyl hydroperoxide by BHT. Most of the apparent inhibition by BHT of non-intact Hb formation in HbCO-containing red cells exposed to t-butyl hydroperoxide is an artefact of haemoglobin loss by haemolysis (Fig. 2*c*).

The influence of glucose metabolism on t-butyl hydroperoxide-induced oxidative alterations is dependent on the level of hydroperoxide exposure. Glucose metabolism via the HMS (Trotta *et al.*, 1982) prevented lipid peroxidation and haemoglobin oxidation in HbO_2- (Fig. 2*a*) and HbCO-containing red cells (Fig. 2*c*) exposed to t-butyl hydroperoxide concentrations up to 0.05 mM. Increasing levels of t-butyl hydroperoxide resulted in lipid peroxidation and oxidation of haemoglobin. At intermediate levels of t-butyl hydroperoxide (0.075 to 0.15 mM) while lipid peroxidation rapidly approaches the levels seen in incubations without glucose, continued glucose protection against haemoglobin oxidation is observed in HbCO-containing red cells (Fig. 2*c*) and to a lesser extent in HbO_2-containing red cells (Fig. 2*a*). In the 90 min incubation time, a significant portion of glucose protection against metHb formation is likely due to metHb-reductase activity after all the t-butyl hydroperoxide has been consumed (see Fig. 1).

In metHb-containing red cells (Fig. 2*b*) we see no significant effect of glucose metabolism on t-butyl hydroperoxide-induced lipid peroxidation. At intermediate levels of t-butyl hydroperoxide (0.025–0.100 mM) the protection of glucose metabolism against haemoglobin oxidation is marginal when compared with metHb-containing red cells incubated without glucose. The formation of HbO_2 observed in metHb-containing red cells exposed to up to 0.100 mM-t-butyl hydroperoxide is due to metHb-reductase activity occurring after t-butyl hydroperoxide has been consumed.

Studies of hydroperoxide-induced lipid peroxidation using a red-cell ghost/metHb/t-butyl hydroperoxide system

In an effort to elucidate the mechanism of t-butyl hydroperoxide-induced lipid peroxidation in red cells, we studied the formation of TBAR and O_2 consumption by red-cell ghosts exposed to t-butyl hydroperoxide and metHb. We utilized an incubation system including 1 μM-metHb and 5% (v/v) red-cell ghosts in phosphate buffer at pH 7.4. There was no significant O_2 consumption by ghosts exposed to t-butyl hydroperoxide in the absence of metHb, indicating that the hydroperoxide cannot induce lipid peroxidation in the absence of a haem catalyst. Fig. 3 shows the dependence of TBAR and O_2 consumption on the t-butyl hydroperoxide concentration. The rates of TBAR and O_2 consumption are linear and directly proportional to the added concentration of t-butyl hydroperoxide. The correlation of O_2 consumption with TBAR suggests that membrane hydroperoxides are breaking down as quickly as they form so that lipid peroxidation is not autocatalytic. At 0.1 mM-t-butyl hydroperoxide both TBAR and O_2 consumption break from linearity at about 25 min. At 0.075 mM-t-butyl hydroperoxide, these parameters break from linearity after about 40 min. At lower levels of added t-butyl hydroperoxide, TBAR and O_2 consumption remain linear for the duration of the incubation. The slowing of the peroxidation rate during incubation occurs at approx. 1400 nM-TBAR and is probably due to consumption of polyenoic fatty acid residues of the ghost membrane phospholipids (Benatti *et al.*, 1981). The results of Fig. 3 yield a molar ratio of O_2

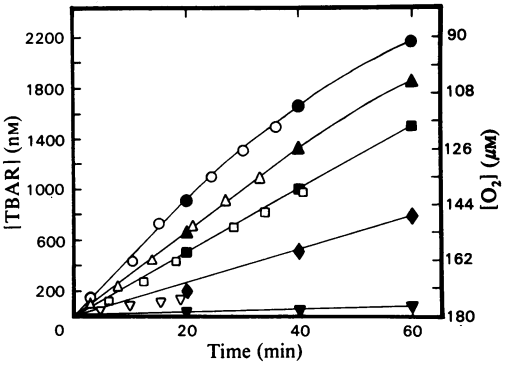

Fig. 3. *Lipid peroxidation, as assessed by measurement of TBAR and O_2 consumption, in red-cell ghosts incubated with metHb and various concentrations of t-butyl hydroperoxide*

A 5% (v/v) red-cell ghost suspension was incubated with 1 μM-metHb and various concentrations of t-butyl hydroperoxide (in mM: 0, ▽ and ▼; 0.025, ♦; 0.050, □ and ■; 0.075, △ and ▲; 0.100, ○ and ●) at 37°C. At various times incubation mixtures were assayed for TBAR (closed symbols) or O_2 consumption (open symbols) as described in the Materials and methods section.

consumed to TBAR of 45 :1. Although almost all O_2 consumption is due to membrane peroxidation, it represents the upper limit of lipid hydroperoxide formation since oxidation of membrane proteins may contribute to O_2 consumption (Logani & Davies, 1980).

Effects of antioxidants on red cells and ghosts

The effects of various antioxidants and free-radical scavengers on lipid peroxidation were compared in two systems: 5% (v/v) red-cell ghosts plus metHb plus t-butyl hydroperoxide, and HbO_2-containing red cells incubated without glucose and exposed to t-butyl hydroperoxide. Incubation conditions were chosen such that there was a linear increase in TBAR with incubation time. Table 7 lists the compounds used, their final concentrations in the incubation and their effects on lipid peroxidation. BHT, a highly efficient terminator of free-radical chain reactions in peroxidizing membrane lipids (Porter, 1980), was highly effective in inhibiting lipid peroxidation in red cells, and 10μM-BHT almost completely suppressed lipid peroxidation in ghosts. Propan-2-ol had no inhibitory effect on peroxidation in ghosts or red cells, arguing against a role for hydroxyl radicals in either system (Dorfman & Adams, 1973). Histidine, a hydroxyl radical scavenger (Dorfman & Adams, 1973), also had no inhibitory effect on lipid peroxidation in either ghosts or intact red cells. Thiourea, which is water-soluble and a hydroxyl radical scavenger (Bartosz & Leyko, 1981), partially inhibited lipid peroxidation in both red cells and ghosts in a concentration-dependent manner. Thiourea, at the higher concentrations used in Table 7, can scavenge t-butoxyl radicals (Bors *et al.*, 1981). DPA, an inhibitor of both lipid peroxidation (Wang & Kimura, 1976) and an excellent scavenger of t-butoxyl radicals (Bors *et al.*, 1981), completely suppressed lipid peroxidation in red cells at lower concentrations than BHT. In the ghost system the opposite was the case. At 50μM, DPA inhibited lipid peroxidation in ghosts by only 44%, whereas 10μM-BHT almost completely suppressed lipid peroxidation. BHT is a very poor scavenger of t-butoxyl radicals compared with DPA (Bors *et al.*, 1981). Urate is an excellent reductant of higher oxidation states of haem-proteins (Howell & Wyngaarden, 1960). Urate at 50μM completely suppresses lipid peroxidation in ghosts, but suppresses lipid peroxidation in red cells by only 25–30%. Ascorbate is an excellent reductant of higher oxidation states of haem-proteins and is an excellent scavenger of t-butoxyl radicals (Bors *et al.*, 1981). Ascorbate at 50μM almost completely inhibited lipid peroxidation in both ghosts and red cells. Urate and ascorbate are not inhibiting lipid peroxidation by scavenging singlet oxygen (Ames *et al.*, 1981) because histidine, another singlet oxygen scavenger, had virtually no effect in both ghosts and red cells. That GSH, which is impermeable to the cell, is able to inhibit strongly lipid peroxidation in red cells suggests that a free radical of low enough reactivity to diffuse through the membrane is generated [i.e., an alkoxy radical (Patterson, 1981)]. This also suggests that GSH protects the red cell from lipid peroxidation non-enzymically by scavenging free-radical initiators. DETAPAC, a pentavalent metal chelator, had only a slight inhibitory effect on lipid peroxidation in both ghosts and intact red cells, indicating no significant role for trace metals such as iron or copper (Barber & Bernheim, 1967).

Table 7. *Effect of antioxidants and free-radical scavengers on lipid peroxidation in red cells and their isolated ghosts exposed to t-butyl hydroperoxide*

A 1% (v/v) suspension of HbO_2-containing red cells was incubated without glucose and exposed to 0.1 mM-t-butyl hydroperoxide; in a separate experiment a 5% (v/v) suspension of red cell ghosts was incubated with 1μM-metHb and exposed to 0.05 mM-t-butyl hydroperoxide. Various antioxidants and free-radical scavengers were included in some experiments as described in the Materials and methods section. All incubations were carried out at 37°C for 40 min followed by measurement of lipid peroxidation. There was negligible lipid peroxidation in incubations without t-butyl hydroperoxide (data not shown).

Additions	Concentration (mM)	Lipid peroxidation as TBAR (nM)* Red cells	Ghosts plus metHb
None		395 (100)	834 (100)
BHT	0.001	308 (78)	672 (81)
	0.01	154 (39)	112 (13)
DPA	0.01	19 (5)	
	0.05	26 (6)	468 (56)
Ascorbate	0.01	142 (36)	580 (70)
	0.05	86 (22)	74 (9)
Urate	0.01	296 (75)	500 (60)
	0.05	277 (70)	86 (10)
DETAPAC	0.03	370 (94)	784 (94)
Glutathione	0.02	241 (61)	672 (81)
	0.1	148 (37)	104 (13)
DPF	0.01	278 (70)	858 (103)
	0.1		734 (88)
Thiourea	1.3	241 (61)	672 (81)
	13.0	68 (17)	240 (29)
Histidine	0.4	360 (91)	932 (112)
Propan-2-ol	130	432 (109)	814 (97)

* Data are the results of a single experiment that is representative of two or three experiments. Data are reported as a percentage of the control (no additions) in parentheses.

Discussion

In red cells exposed to t-butyl hydroperoxide, lipid peroxidation and haemoglobin degradation represent extremes of a spectrum of oxidative damage (Trotta *et al.*, 1981, 1982). Reduction of t-butyl hydroperoxide by GSH peroxidase results in sparing of membrane lipids from peroxidation. Consumption of t-butyl hydroperoxide by reaction with haemoglobin results in the generation of initiators of lipid peroxidation. The degree of lipid peroxidation is greatly dependent on the haemoglobin status. HbO_2 and HbCO are excellent initiators of lipid peroxidation, as are low concentrations of metHb. Higher concentrations of metHb effect reduction of t-butyl hydroperoxide more rapidly than HbO_2 or HbCO and appear to scavenge efficiently any free-radical products of the reduction and spare the membrane from peroxidation. These findings led to the development of a hypothesis about casual relationships between lipid peroxidation, haemoglobin oxidation and degradation, and increased flux through the HMS in red cells exposed to t-butyl hydroperoxide (Trotta *et al.*, 1981, 1982). In the present study, the use of BHT as an inhibitor of the propagative events of lipid peroxidation indicates that the bulk of the consumption of t-butyl hydroperoxide takes place by direct interaction between haem and t-butyl hydroperoxide and between GSH and t-butyl hydroperoxide via GSH peroxidase. For example, in HbO_2-containing red cells in the presence of glucose and 0.1 mM-t-butyl hydroperoxide, 25 μM-BHT inhibited formation of products of lipid peroxidation by 79% whereas total haemoglobin oxidation and degradation was slightly but significantly decreased from 38% to 33% with no significant effect on flux through the HMS. These findings show that the presence or absence of lipid peroxidation has only minor effects on the overall metabolism of t-butyl hydroperoxide, whereas, in contrast, the rate and extent of lipid peroxidation is dependent on the haem state and the glucose flux through the HMS (Trotta *et al.*, 1982).

Total haemoglobin oxidation and degradation were slightly but significantly decreased in the presence of BHT. BHT-protection of haemoglobin was between 2 and 8% of total red-cell haem, depending on the haem state at zero time and the presence or absence of glucose. Hydroperoxides could not be detected in red-cell membrane lipids after exposure of red cells to t-butyl hydroperoxide, whether or not the red cells were incubated with glucose, suggesting that hydroperoxides were being broken down by a glucose-independent mechanism. These observations suggest that haemoglobin readily reacts with and decomposes membrane lipid hydroperoxides. Consistent with this hypothesis is the observation that BHT inhibited the binding of haemoglobin metabolites to the membrane, especially in the absence of glucose. The extremely hindered phenolic group of BHT renders it unreactive with haemoglobin, making it unlikely that a direct interaction between haemoglobin and BHT could prevent t-butyl hydroperoxide-induced haemoglobin alterations. Cross-linking of haemoglobin to the membrane by malonaldehyde (the major component of TBAR) was probably not significant, since concentrations of TBAR were too low and the incubation period was too short for this process to contribute significantly to loss of haemoglobin (Goldstein *et al.*, 1980).

Haem or haem-promoted membrane hydroperoxide decomposition appears to be more important than an enzymic pathway via GSH peroxidase. In the presence of concentrations of BHT that inhibited 70–80% of lipid peroxidation, significant inhibition of HMS activity would be expected if GSH peroxidase utilized membrane lipid hydroperoxides as substrates. Experimentally we observed only a 5% decrease in HMS activity in metHb-containing red cells and no significant effects in HbO_2- and HbCO-containing red cells. The possibility that GSH peroxidase has a role in the metabolism of lipid hydroperoxides under more physiological conditions, i.e. when a high concentration of another competing hydroperoxide (t-butyl hydroperoxide) is not present, is not excluded by this experimental model. The hypothesis that selenium-dependent GSH peroxidase (the only GSH peroxidase in red cells) plays a role in reduction of membrane lipid hydroperoxides (Christopherson, 1969; Tappel, 1980) has come under question since the demonstration that selenium-dependent GSH peroxidase does not utilize phospholipid-bound fatty acid hydroperoxides as substrates (McCay *et al.*, 1976; Stults *et al.*, 1977; Burk *et al.*, 1980; Gibson *et al.*, 1980; Ursini *et al.*, 1982) whether they are in free solution or incorporated into lipid bilayers.

With red cells containing HbO_2 or metHb or red cells containing HbCO in the absence of extracellular glucose, the rate of formation of TBAR decreases to zero when t-butyl hydroperoxide disappears from the medium. Hydroperoxides could not be detected in the extracted membrane lipids of t-butyl hydroperoxide-exposed HbCO-containing red cells incubated with or without glucose at either 20 or 60 min (data not shown). This implies that the propagative and terminative reactions of lipid peroxidation are relatively rapid in this biological model in comparison with the initiation reactions supported by t-butyl hydroperoxide. The propagative and terminative phases are shortened by the availability of oxy-, deoxy-, and met-haem groups. The only exception to this finding is seen with red cells containing HbCO in the presence of glucose, in

which the formation of significant levels of metHb or non-intact Hb is significantly delayed. In this preparation formation of TBAR continues at a significant rate after the disappearance of t-butyl hydroperoxide from the medium. From these results it appears that non-cellular models of lipid peroxidation are most closely approximated by the blocking of haem reactivity by CO and prevention of met-haem accumulation by glucose metabolism.

The ability of the HMS to compete with haemoglobin for t-butyl hydroperoxide and therefore prevent haemoglobin oxidation and concomitant lipid peroxidation is dependent on the reactivity of haemoglobin towards t-butyl hydroperoxide, which follows the order metHb > HbO_2 > HbCO. At high levels of t-butyl hydroperoxide (0.200 to 0.250 mM) glucose metabolism was probably saturated and differences in lipid peroxidation and haemoglobin alterations were insignificant or marginal when comparing incubations with and without glucose. Under these extreme oxidative conditions, accumulation of metHb determined protection from lipid peroxidation. Red cells slower to accumulate metHb underwent higher levels of lipid peroxidation.

The HbCO-containing red cells are slowest to form metHb and non-intact Hb and they undergo the highest levels of lipid peroxidation and also haemolysis. Haemolysis occurs after a level of about 800 nM-TBAR has been reached. A similar relationship between lipid peroxidation and haemolysis was observed when red cells were exposed to H_2O_2 plus azide (Stocks & Dormandy, 1971) or phenyldiazene (Goldberg & Stern, 1977). The antioxidant BHT inhibited lipid peroxidation by about 50% and virtually completely inhibited haemolysis. HbCO-containing red cells exposed to high concentrations of t-butyl hydroperoxide underwent less haemoglobin oxidation and precipitation and more lipid peroxidation (with concomitant haemolysis) than HbO_2- and metHb-containing red cells (which did not haemolyse). These results suggest that oxidative membrane damage is a more important determinant of haemolysis than haemoglobin degradation in this model.

The results with free-radical scavengers are consistent with an important role for t-butoxyl radicals as initiators of lipid peroxidation in red cells exposed to t-butyl hydroperoxide. The single electron oxidation of HbO_2 (and HbCO) to metHb suggests that the homolytic scission of the O—O bond of t-butyl hydroperoxide has occurred, yielding t-butoxyl radical (t-BuO$^{\bullet}$) (Trotta *et al.*, 1982). The reaction of metHb with t-butyl hydroperoxide may result in both formation of t-BuO$^{\bullet}$ and ferryl haem (haem[Fe(IV) = O]). Ferryl haem may also contribute to initiation of lipid peroxidation (George, 1953). The products of the haem-promoted decomposition of t-butyl hydroperoxide are most likely

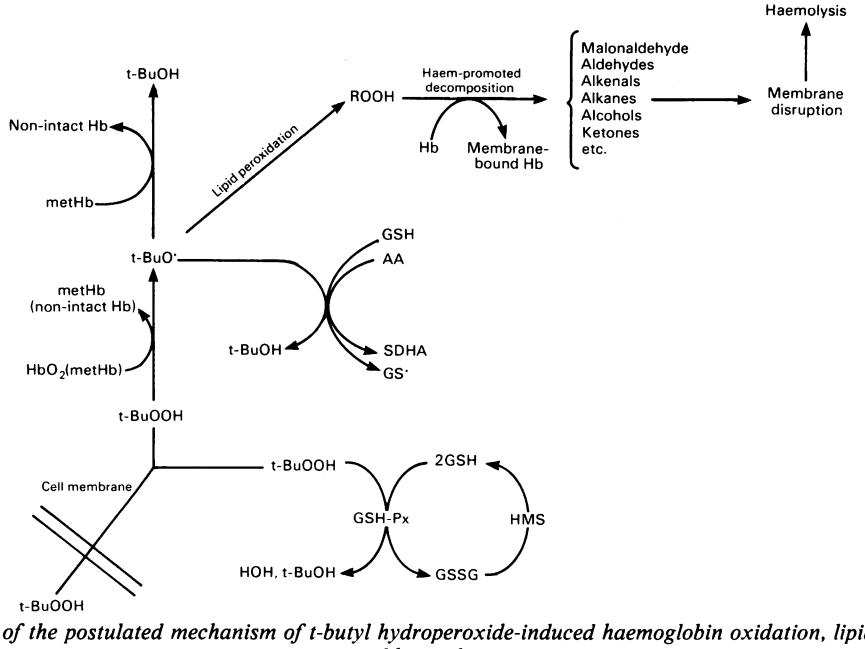

Fig. 4. *Scheme of the postulated mechanism of t-butyl hydroperoxide-induced haemoglobin oxidation, lipid peroxidation and haemolysis*

Abbreviations used: t-BuOOH, t-butyl hydroperoxide; t-BuOH, t-butyl alcohol; t-BuO$^{\bullet}$, t-butoxyl radical; AA, reduced ascorbate; SDHA, semidehydroascorbate; GSH-Px, glutathione peroxidase; GSSG, oxidized glutathione; GS$^{\bullet}$, glutathionyl radical; ROOH, lipid hydroperoxide.

generated in close proximity to the membrane. In red cells, only a small fraction of the total haemoglobin concentration is in close apposition to the membrane and higher oxidation states of haem are probably formed to a lesser extent than in ghosts, diminishing the relative importance of a direct interaction of highly oxidized haemoglobin with the membrane as a mechanism of initiation of lipid peroxidation in intact red cells. Hence DPA was observed to be a highly effective lipid-peroxidation inhibitor in the red cell and less so in ghosts. Consistent with our results suggesting haemoglobin-promoted decomposition of t-butyl hydroperoxide to free radicals are the results of Griffin & Ting (1978), where in the reaction of metmyoglobin with cumene hydroperoxide oxidative destruction of the haem groups and production of the cumyloxyl radical is observed.

We now propose a scheme for t-butyl hydroperoxide-induced lipid peroxidation in red cells (Fig. 4). t-Butyl hydroperoxide diffuses freely into the red cell cytosol, where it is reduced either enzymically by GSH peroxidase or via free-radical-mediated decomposition by haemoglobin. Enzymic reduction of t-butyl hydroperoxide prevents both haemoglobin oxidation and lipid peroxidation and results in oxidation of NADPH and stimulation of the HMS (Oshino & Chance, 1977; Trotta *et al.*, 1982). Reaction of t-butyl hydroperoxide with haemoglobin results in formation of t-butoxyl radicals, which then react with membrane lipids to initiate peroxidation (Patterson, 1981). The lipid hydroperoxides (ROOH) that form appear not to be utilized as substrates by GSH peroxidase but may react with haemoglobin, resulting in their decomposition to various end-products, including malonaldehyde (Barber & Bernheim, 1967), alkenals, aldehydes, ketones and hydrocarbon gases (Esterbauer, 1982). Extensive lipid peroxidation results in membrane disruption and haemolysis (Kunimoto *et al.*, 1981). Lipid peroxidation may be prevented by scavenging of t-butoxyl radicals by metHb with conversion into non-intact Hb. Hydroperoxide-derived free radicals readily react with haemoproteins and result in their oxidative degradation (O'Brien, 1969). Cellular ascorbate and GSH protect the membrane by rapidly scavenging t-butoxyl radicals and are maintained in reduced form by reducing equivalents from both the HMS and glycolysis (Trotta *et al.*, 1982).

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