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Oxidative Insult in Sheep Red Blood Cells Induced by T-Butyl Hydroperoxide: The Roles of Glutathione and Glutathione Peroxidase

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Three different types of red blood cells (RBC) were used:(i) RBC from sheep having genetically high GSH (ii) RBC from sheep with genetically low GSH and (iii) RBC from high-GSH sheep treated with CDNB to deplete GSH. Incubation of these RBC with t-butyl hydroperoxide (tBHP, 3mM) for 10 min caused the formation of TBARS, oxidation of haemoglobin and degradation and aggregation of membrane proteins in RBC from low-GSH sheep and GSH-depleted RBC. By contrast, RBC from high-GSH sheep(normal RBC) did not show the degradation and aggregation of membrane proteins within the first 10 min. Dithiothreitol (DTT) was highly effective in preventing the tBHP-mediated oxidation of haemoglobin, the formation of TBARS and the degradation and aggregation of membrane proteins in both normal RBC and low-GSH RBC. However, DTT did not provide protection in GSH-depleted RBC or normal RBCs in the presence of 1.5 mM mercaptosuccinate (MCS), a potent inhibitor of GSH peroxidase (GSHPx). The ability of GSH to prevent the oxidation of haemoglobin and the degradation and aggregation of membrane proteins was abolished in the presence of MCS. These results indicate that the protective function of DTT involves a GSH-dependent mechanism. Both GSH and GSHPx play key roles in this enzymatic system. In the light of the complete protection of RBC against oxidation induced by tBHP in the presence of DTT or GSH, the GSH/GSHPx system appears to act directly as a tBHP scavenger. The activities of four well-known antioxidants, Butylated hydroxytoluene, ascorbate, α-tocopherol and desferrioxamine were also tested in this study to cast further light on the role of free radical scavenging in protection from tBHP mediated free radical insult.

Keywords: Glutathione, Glutathione peroxidase, Oxidation, t-Butyl hydroperoxide, Red blood cell

Abbreviations: GSH, glutathione, GSHPx, glutathione peroxidase, RBC, red blood cells, tBHP, t-butyl hydroperoxide, DTT, dithiothreitol, BHT, butylated hydroxytoluene, DFO, desferrioxamine, CNDB, 1-chloro-2,4-dinitrobenzene, MCS, mercaptosuccinate, HCT, haematocrit, Hb, haemoglobin, OxyHb, oxyhaemoglobin, MetHb, methaemoglobin, TBARS, thiobarbituric acid-reactive substances, MDA, malonyldialdehyde, HMWP, high molecular weight proteins, LMHP, low molecular weight proteins, t-BuO·, alkoxyl radical, t-BuOO·, alkyl peroxyl radical

INTRODUCTION

Although the erythrocyte is continuously exposed to oxidants such as hydrogen peroxide and lipid peroxides, it maintains its normal structure and functions under physiological conditions. This may be attributed to its effective

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antioxidation systems including enzymes such as catalase, glutathione peroxidase (GSHPx) and superoxide dismutase (SOD) [1] as well as low molecular weight antioxidants such as reduced glutathione (GSH) [2]. Erythrocyte GSH has long been considered to serve a predominant role as a reducing agent responsible for defence of red blood cells (RBC)against oxidative damage [2–5]. In human beings, hereditary deficiencies in the enzymes of GSH synthesis are often associated with the occurrence of haemolytic anemia [6]. There are certain breeds of sheep that have an inherited low GSH in RBC (about 20 % of normal sheep). It has been shown that these low-GSH sheep RBC are more susceptible to S-methylcysteine sulfoxide than those of normal GSH sheep RBC [7]. Furthermore, a high negative correlation was observed between Heinz body formation and GSH level in low-GSH sheep RBC exposed to acetylphenylhydrazine [8]. On the other hand, sheep with low RBC GSH do not show haemolytic diseases [9,10]. Furthermore, some studies indicate that GSH itself may be dispensable for erythrocyte defense against major forms of oxidant damage [11,12].

Thus the knowledge concerning the role of GSH in sheep RBC is far from complete. In this study, we used two types of genetically inherited sheep RBC, low-GSH RBC and high-GSH RBC (normal RBC) as well as GSH (chemically)-depleted normal RBC as models to investigate the oxidative effects of *t*-butyl hydroperoxide (tBHP) on the oxidation of haemoglobin (Hb), lipid peroxidation and the degradation of membrane proteins. Previous studies demonstrated that tBHP induced lipid peroxidation [13,14], oxidation of Hb[13,14], changes in membrane proteins [15,16], leakage of K^+ [17], and inhibition of Ca^{2+}-Mg^{2+}-ATPase [18]. Since tBHP itself is a substrate of GSHPx, we assessed the roles of GSH and GSHPx in preventing oxidative damage induced by tBHP. Given the uncertainty regarding the exact nature of the free radicals involved in tBHP damage, another aim of this study was to investigate the effects of several well-described antioxidants with different mechanisms of action, butylated hydroxytoluene (BHT), ascorbate, α-tocopherol and desferrioxamine (DFO).

MATERIALS AND METHODS

tBHP, 5,5-dithiobis (2-nitrobenzoic acid) (DNTB), GSH, 1-chloro-2,4-dinitrobenzene (CDNB), mercaptosuccinate (MCS) and BHT were purchased from Sigma Co. Australia. Dithiothreitol (DTT), DFO, α-tocopherol and ascorbate were purchased from ICN Co., Australia. All other chemicals were analytical reagent grade.

Blood was obtained from high-GSH and low-GSH sheep by venipuncture into heparinised tubes. RBC were washed three times in phosphate-buffered saline (PBS) consisting of 135 mM NaCl and 10 mM sodium phosphate, pH 7.4. The washed RBC were suspended in PBS to a final haematocrit (HCT) of 10 %. GSH-depleted RBC were prepared by incubating RBC from high-GSH sheep with CDNB according to the method of Awasthi et al [19]. Washed RBC from high-GSH sheep were suspended in PBS to yield HCT of 30% and were incubated with 3 mM CDNB for 30 min at 37°C. The cells were then washed five times to remove unreacted CNDB. The GSH-depleted RBC were re-suspended to a final HCT of 10 %. To prepare hypotonic lysates, 5 ml of RBC (HCT 10 %) were centrifuged at 2000 × g for 5 min and the supernatants removed. The cells were lysed in 5 ml of 10 mM phosphate buffer, pH 7.4 and vortexed. Intact RBC or lysates were preincubated for 30 min with the chemicals tested before tBHP was added.

Membranes were prepared from the reaction mixtures (either lysates or whole cells) according to the method of Dodge et al [20]. Membrane protein concentration was assayed by the method of Lowry et al [21]. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according

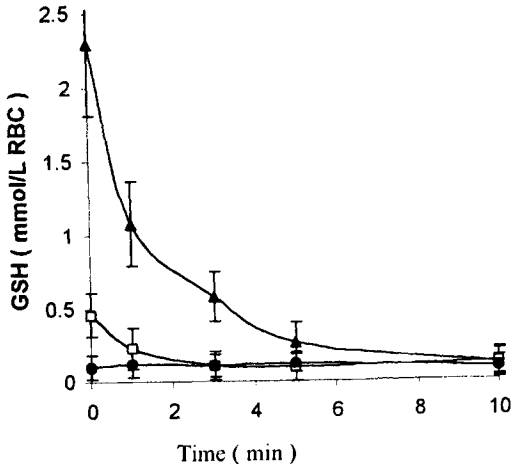


FIGURE 1 Loss of GSH induced by tBHP in sheep RBC. 10 % suspensions of RBC were incubated with 3 mM tBHP at 37°C RBC from High-GSH sheep (filled triangles, H); RBC from low-GSH sheep (empty squares, L); GSH-depleted RBC (filled circles, D). Data are the means ± SD of four separate experiments

to the procedure of Laemmli [22]. The slab gel consisted of a 10 % separating gel and a 4 % stacking gel. Protein bands were visualized by staining with Coomassie brilliant blue.

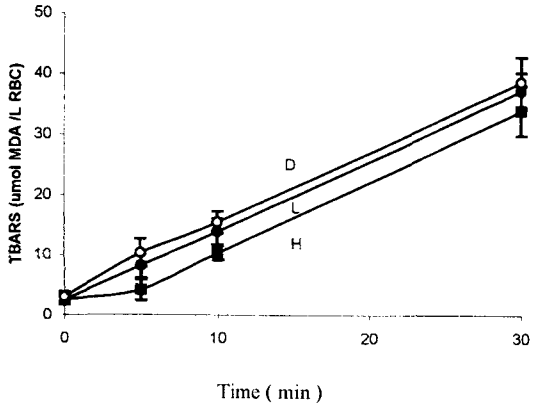


FIGURE 2 Formation of TBARS induced by tBHP in sheep RBC. 10 % suspensions of RBC were incubated with 3 mM tBHP at 37°C. RBC from High-GSH sheep (filled squares, H); RBC from low-GSH sheep (filled squares, L); GSH-depleted RBC (empty circles, D). Data are the means ± SD of four separate experiments

GSH was assayed according to the colourimetric method by evaluating the reduction of 5,5′-dithiobis(2-nitrobenzoic acid) (DNTB) at 412 nm[23]. The concentrations of OxyHb, MetHb and haemichrome were determined by the method of Szebeni et al [24]. Thiobarbituric acid-reactive substances (TBARS), were assayed as described by Stocks and Dormandy[25]. Results of assays comparing treated cells with control samples (treated with tBHP alone) were analyzed for statistical significance using two tailed paired Student t-test.

RESULTS

Loss of GSH

CDNB treatment of RBC from high-GSH sheep resulted in almost 95 % depletion of GSH giving rise to the GSH-depleted RBC used as an experimental model here (Fig. 1, solid circles, zero time point). As shown in Fig. 1, exposure of sheep RBC to tBHP resulted in rapid depletion of GSH. The decrease in GSH level in the first 5 min was from 2.29 ± 0.48 mM to 0.24 ± 0.14 mM in RBC from high-GSH sheep and from 0.48 ± 0.14 mM to 0.12 ± 0.09 mM in RBC from low-GSH sheep (Fig. 1). The value reached a common basal level in RBC from high and low-GSH sheep, respectively over 10 min reflecting the level in GSH depleted RBC. In fact, there was little further change in GSH levels and the values at 30 min were almost same as those at 10 min in all three types of cells.

Formation of TBARS

The extent of lipid peroxidation, after exposure to tBHP, was monitored by the production of TBARS. As shown in Fig. 2, TBARS increased in a time-dependent manner. There was a notable significant lag phase in the formation of TBARS in RBC from high-GSH sheep by comparison with those of RBC from low-GSH sheep or

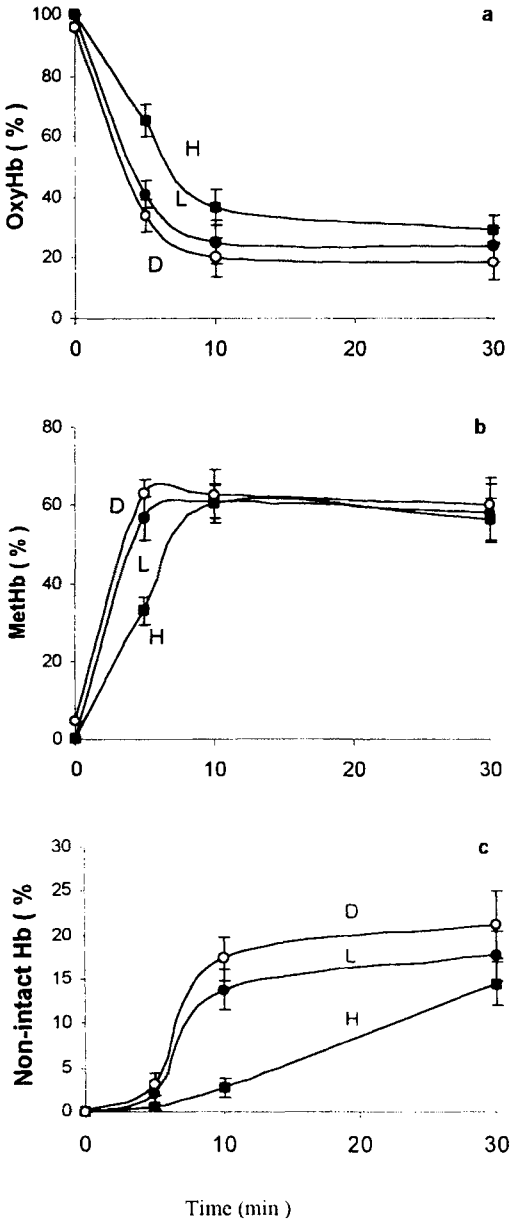


FIGURE 3 Changes in haemoglobin status induced by tBHP in sheep RBC. a) OxyHb; b) MetHb; c) Haemichrome 10 % suspensions of RBC were incubated with 3 mM tBHP at 37°C. RBC from High-GSH sheep (filled squares, H); RBC from low-GSH sheep (filled squares, L); GSH-depleted RBC (empty circles, D). Data are the means ± SD of four separate experiments

GSH-depleted RBC after 5 min incubation. There were however no significant differences in these three types of RBC following incubation with tBHP after 30 min.

Oxidation of Haemoglobin (Hb)

Fig. 3 shows the changes in haemoglobin status in RBC exposed to tBHP. Incubation of RBC with tBHP resulted in a decrease in OxyHb (Fig. 3a). The concentration of OxyHb rapidly decreased to almost the lowest levels at 10 min of incubation in all three types of RBC. At 5 min, however, the content of OxyHb in RBC from high-GSH sheep was significantly higher than that in the other two types of RBC. There were no significant differences in OxyHb levels among the three types of RBC at 30 min. Reciprocal changes occurred in the levels of MetHb (Fig 3b). There was a rapid rise in MetHb in RBC from low-GSH sheep as well as the GSH-depleted RBC in the first 5 min of incubation. The level of MetHb reached only half of its maximum in RBC from high-GSH sheep over the same time period. There were no significant differences among the three types of RBC after 10 min incubation. The levels of haemichrome in the three types of RBC were very low in the first 5 min incubation with tBHP (Fig 3c). While there was a rapid rise in the levels of haemichrome at 10 min in RBC from genetically low-GSH sheep and normal GSH-depleted RBC, the rise in haemichrome in RBC from normal high-GSH sheep was comparatively very moderate. After 30 min of incubation, the rise in haemichrome levels occurred in all three types of cells.

Degradation and Aggregation of Membrane Proteins

tBHP treatment clearly resulted in damage to RBC cytoskeletal proteins. As shown in Fig. 4 (Lanes 4–7), these changes included mainly the formation of high molecular weight polymers

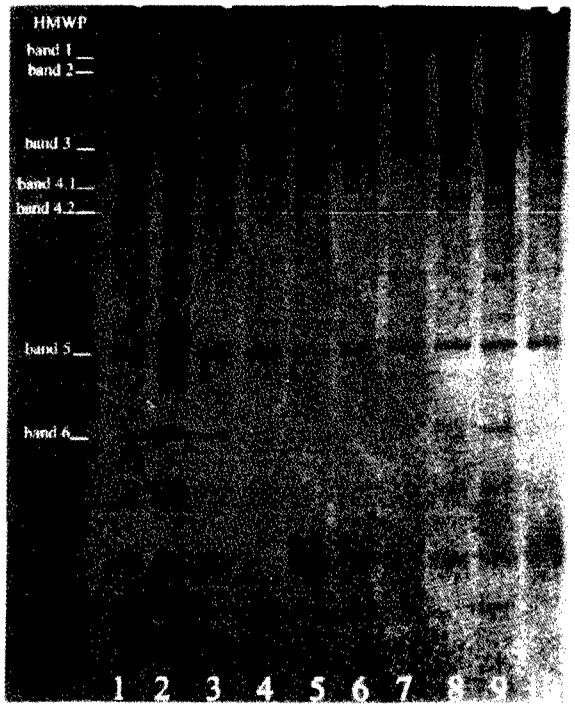


FIGURE 4 tBHP-mediated degradation and aggregation of membrane proteins in sheep RBC. SDS-PAGE separation of membrane proteins in sheep RBC was achieved by the method of Laemmli [22]. Lanes 1,2,3, membrane proteins from High-GSH, Low-GSH and GSH-depleted RBC, respectively without tBHP; lanes 4,5,6, membrane proteins from High-GSH, Low-GSH and GSH-depleted RBCs respectively incubated with 3 mM tBHP for 10 min at 37°C; lane 7, membrane proteins from high-GSH RBC incubated with 3 mM tBHP for 30 min at 37°C; lanes 8, 9, 10, membrane proteins from High-GSH, Low-GSH and GSH-depleted RBC respectively, preincubated with 5 mM DTT prior to tBHP (See Color Plate V at the back of this issue)

(HMWP) that appear in the stacking gel, the loss of spectrins (band 1 and band 2), band 3, band 4.1 and band 4.2 and the appearance of low molecular weight proteins (LMWP). These changes occurred in RBC from low-GSH and GSH-depleted RBC after 10 min (Fig. 4, Lanes 5,6). Only slight decrease in spectrins and band 3 were observed in high-GSH RBC after 10 min (Fig. 4, Lane 4). Complete degradation and aggregation of membrane proteins did occur in high-GSH RBC but only after 30 min of incubation (Fig. 4, Lane 7).

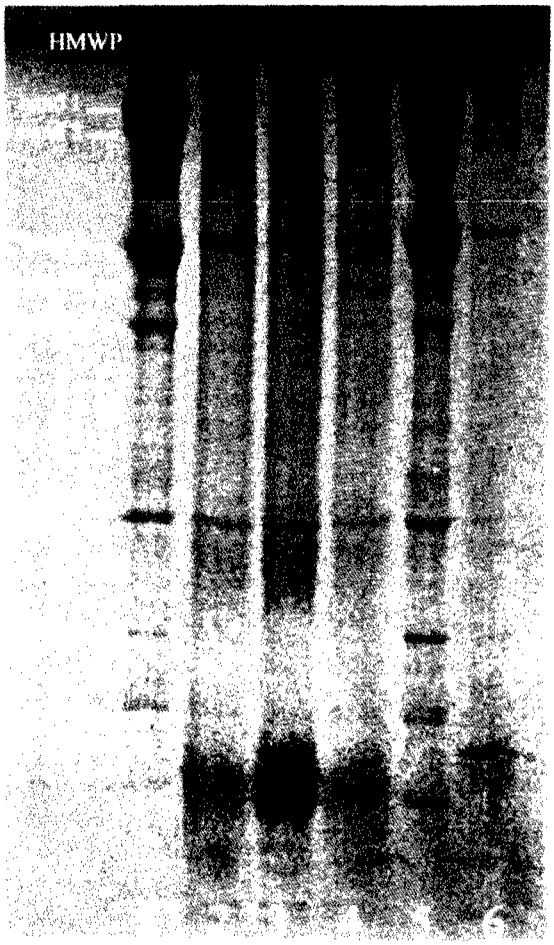
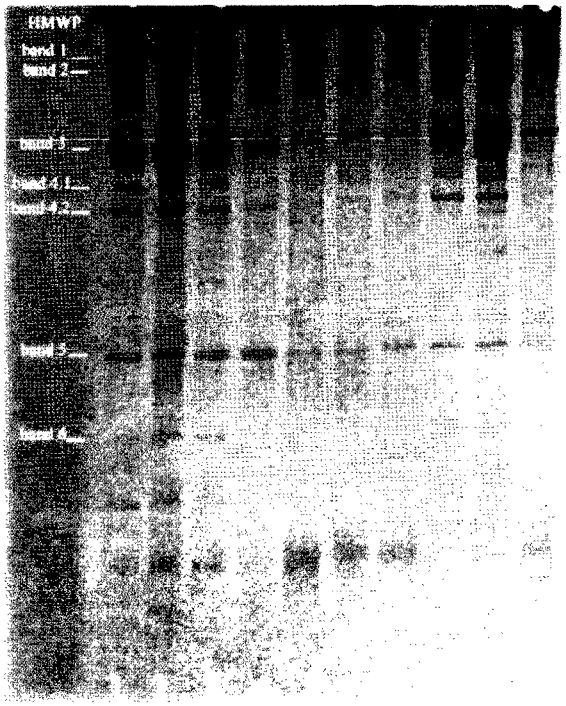


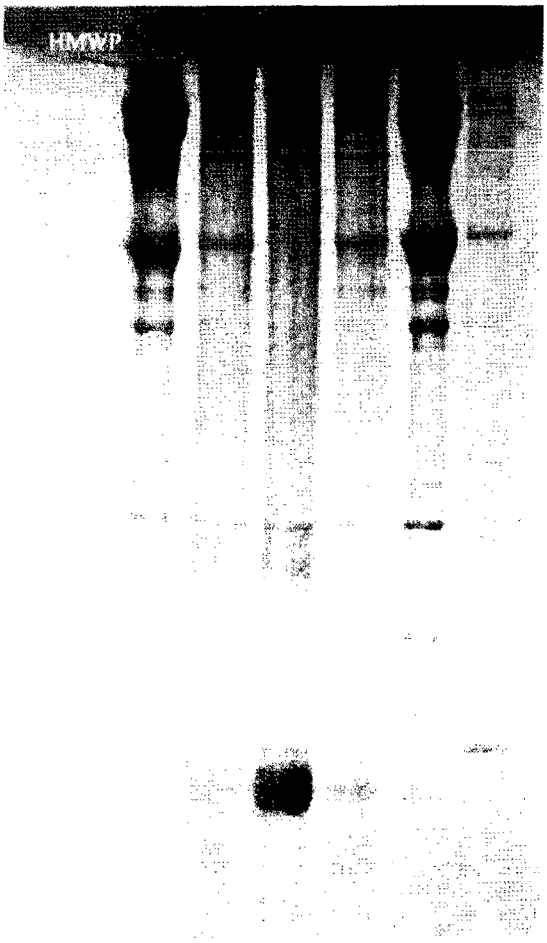
FIGURE 5 The roles of GSH and GSHPx on tBHP-mediated Degradation and aggregation of membrane proteins in normal RBC. Lane 1, membrane proteins from normal RBC without tBHP; lane 2, membrane proteins from normal RBCs incubated with tBHP; lane 3, membrane proteins from normal RBCs preincubated with 1.5 mM MCS and 5 mM DTT prior to tBHP; lane 4; membrane proteins from normal RBCs preincubated with 5 mM GSH prior to tBHP; lane 5, membrane proteins from normal RBC lysates preincubated with 5 mM GSH prior to tBHP; lane 6, membrane proteins from normal RBC lysates preincubated with 1.5 mM MCS and 5 mM GSH prior to tBHP (See Color Plate VI at the back of this issue)

Protection by DTT Against Oxidation Induced by tBHP

Table I shows that DTT is very effective in preventing the formation of TBARS and inhibiting the oxidation of haemoglobin in both RBC from



Color Plate V (See page 49, Figure 4) tBHP-mediated degradation and aggregation of membrane proteins in sheep RBC. SDS-PAGE separation of membrane proteins in sheep RBC was achieved by the method of Laemmli [22]. Lanes 1,2,3, membrane proteins from High-GSH, Low-GSH and GSH-depleted RBC, respectively without tBHP; lanes 4,5,6, membrane proteins from High-GSH, Low-GSH and GSH-depleted RBCs respectively incubated with 3 mM tBHP for 10 min at 37°C; lane 7, membrane proteins from high-GSH RBC incubated with 3 mM tBHP for 30 min at 37°C; lanes 8, 9, 10, membrane proteins from High-GSH, Low-GSH and GSH-depleted RBC respectively, preincubated with 5 mM DTT prior to tBHP



Color Plate VI (See page 49, Figure 5) The roles of GSH and GSHPx on tBHP-mediated Degradation and aggregation of membrane proteins in normal RBC. Lane 1, membrane proteins from normal RBC without tBHP; lane 2, membrane proteins from normal RBCs incubated with tBHP; lane 3, membrane proteins from normal RBCs preincubated with 1.5 mM MCS and 5 mM DTT prior to tBHP; lane 4; membrane proteins from normal RBCs preincubated with 5 mM GSH prior to tBHP; lane 5, membrane proteins from normal RBC lysates preincubated with 5 mM GSH prior to tBHP; lane 6, membrane proteins from normal RBC lysates preincubated with 1.5 mM MCS and 5 mM GSH prior to tBHP

high-GSH and low-GSH sheep even at 30 min incubation when GSH levels were almost completely depleted in the absence of DTT in all three .cell types. (The GSH content in DTT treated RBC could not be determined accurately due to interference from DTT.) DTT also effectively protected membrane proteins against degradation and aggregation induced by tBHP (Fig. 4, lanes 8,9). Interestingly, DTT did not prevent either the oxidation of haemoglobin or the degradation and aggregation of membrane proteins or the formation of TBARS so well in GSH-depleted RBC (Table I and Fig. 4, lane 10). There were no differences between intact GSH-depleted RBC and lysates made from the same RBC (data not shown). These data demonstrate that the action of DTT is effective only when a certain (albeit low) level of GSH exists (e.g. RBC from low-GSH sheep) but is ineffective if almost no GSH exists in a system (e.g. GSH-depleted RBC). Taken together, these results strongly suggest that protection of DTT against oxidation caused by tBHP is GSH-dependent.

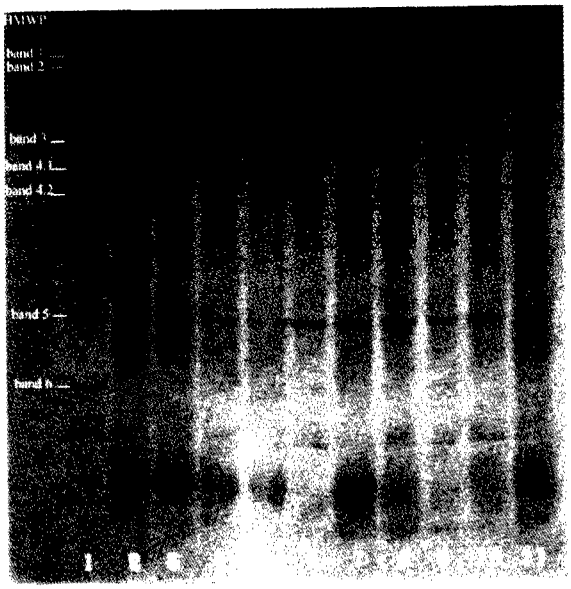


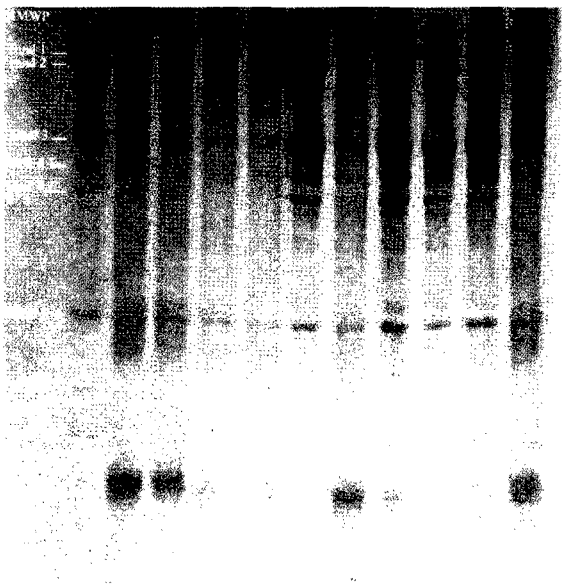
FIGURE 6 The effects of antioxidants on the degradation and aggregation of RBC membrane proteins induced by tBHP. RBC and lysates were preincubated with antioxidant prior to tBHP. 10 % of RBC suspensions or lysates were incubated with 3 mM tBHP for 30 min at 37°C. Lane 1, membrane proteins from normal RBCs without tBHP; lane 2, membrane proteins from normal RBC incubated with tBHP alone; lanes 3, 4, membrane proteins from normal RBC and lysates respectively in the presence of 100 μM BHT and tBHP; lanes 5, 6, membrane proteins from normal intact RBC and lysates respectively in the presence of 5 mM ascorbate and tBHP; lanes 7, 8, membrane proteins from normal intact RBC and lysates respectively in the presence of 5 mM DFO and tBHP; lane 9, membrane proteins from lysates in the presence of 5 mM DFO, 5 mM ascorbate and tBHP; Lanes 10, 11, membrane proteins from normal intact RBC and lysates respectively in the presence of 5 mM α-tocopherol and tBHP (See Color Plate VII at the back of this issue)

Role of GSH and GSHPx in Oxidation Caused by tBHP

When normal RBC (i.e. from high-GSH sheep) were preincubated with MCS, a potent inhibitor of GSHPx [26], we found that DTT failed to prevent the formation of TBARS, the oxidation of haemoglobin and the degradation and aggregation of membrane proteins even though an adequate supply of GSH was available (Table II and Fig. 5, Lane 3).

To provide more direct evidence, added GSH was tested in further experiments. As shown in Table II and Fig. 5, incubation of normal RBC with 5 mM GSH added prior to the addition of tBHP could not prevent the formation of TBARS, the oxidation of haemoglobin and degradation and aggregation of membrane proteins (Table II and Fig. 5, Lane 4). If however lysates were incubated with GSH, GSH was as effective as DTT (Table II, Fig. 5, Lane 5).

These result may be explained by assuming that GSH could not be transported into RBC in the intact RBC. The result further implied that GSH was ineffective in preventing tBHP-induced oxidation if it could not be utilized as a substrate by GSHPx. To test this hypothesis, incubation of GSH with the lysates prepared from GSHPx-inhibited RBC prior to tBHP showed that GSH by itself had no effect on the formation of TBARS, the oxidation of haemoglobin and degradation and aggregation of membrane proteins induced by tBHP (Table II and Fig. 5, Lane 6).



Color Plate VII (See page 50, Figure 6) The effects of antioxidants on the degradation and aggregation of RBC membrane proteins induced by tBHP. RBC and lysates were preincubated with antioxidant prior to tBHP. 10 % of RBC suspensions or lysates were incubated with 3 mM tBHP for 30 min at 37°C. Lane 1, membrane proteins from normal RBCs without tBHP; lane 2, membrane proteins from normal RBC incubated with tBHP alone; lanes 3, 4, membrane proteins from normal RBC and lysates respectively in the presence of 100 μM BHT and tBHP; lanes 5, 6, membrane proteins from normal intact RBC and lysates respectively in the presence of 5 mM ascorbate and tBHP; lanes 7, 8, membrane proteins from normal intact RBC and lysates respectively in the presence of 5 mM DFO and tBHP; lane 9, membrane proteins from lysates in the presence of 5 mM DFO, 5 mM ascorbate and tBHP; Lanes 10, 11, membrane proteins from normal intact RBC and lysates respectively in the presence of 5 mM α-tocopherol and tBHP

TABLE I The effect of DTT on the oxidation induced by tBHP in sheep RBC

Sample	*GSH (mmol/L RBC)*	*TBARS (μmol MDA/L RBC)*	*MetHb (%)*	*OxyHb (%)*	*Haemichrome (%)*
H + tBHP	0.10 ± 0.08	32.82 ± 4.28	56.2 ± 6.8	29.3 ± 3.5	14.5 ± 3.8
L + tBHP	0.11 ± 0.05	35.63 ± 5.27	60.2 ± 7.4	24.1 ± 4.7	15.7 ± 4.9
D + tBHP	0.08 ± 0.05	34.07 ± 3.48	60.1 ± 5.5	20.8 ± 4.3	19.1 ± 6.3
H + DTT + tBHP	ND	6.88 ± 1.38****	0.8 ± 0.2****	98.8 ± 1.1****	0.4 ± 0.1****
L + DTT + tBHP	ND	7.28 ± 1.27****	1.2 ± 0.3****	97.3 ± 1.0****	1.5 ± 0.3****
D + DTT + tBHP	ND	24.23 ± 4.21*	57.2 ± 10.8	25.3 ± 5.1	17.5 ± 3.5

Note. H; High-GSH RBC i.e. high GSH RBC
L; low-GSH RBC.D; GSH-depleted RBC.
ND; Not determined.Different from RBC with 3mM tBHP alone (*P<0.05; **P<0.01; ***P< 0.005; **** P< 0.001)
RBC suspension (HCT 10 %) was incubated with 3 mM tBHP for 30 min at 37°C If DTT or GSH was used in these experiment, 5 mM DTT was added prior to tBHP in these systems. The data represent mean ± SD of four separate experiments.

TABLE II The role of GSH and GSHPx on the oxidation of RBC induced by tBHP

Sample	*GSH (mmol/L RBC)*	*TBARS (μmol MDA/L RBC)*	*MetHb (%)*	*OxyHb (%)*	*Haemichrome (%)*
RBC	2.03 ± 0.31	5.12 ± 1.47	0.2 ± 0.2	100	0
RBC + tBHP	0.11 ± 0.07	30.57 ± 3.48	54.6 ± 6.4	25.5 ± 3.6	19.8 ± 2.8
RBC + MCS + DTT + tBHP	ND	24.92 ± 4.32	55.8 ± 10.9	27.4 ± 4.3	16.8 ± 3.4
RBC + GSH + tBHP	ND	27.28 ± 2.10	54.6 ± 7.8	30.5 ± 4.3	15.0 ± 2.8
Lysates + GSH + tBHP	ND	7.52 ± 2.17****	2.8 ± 0.5****	97.2 ± 1.0****	0****
(Lysates + MCS) + GSH + tBHP	ND	26.69 ± 2.87	57.4 ± 9.5	23.8 ± 3.6	18.8 ± 2.9

Note. RBC; normal RBC.
ND; Not determined.Lysates; the lysates was prepared by lysis of normal RBC in 10 mM phosphate buffer (pH 7.4) (Lysates + MCS); the lysates was prepared by GSHPx inhibition RBC (i.e. normal RBC treated by MCS) lysis in 10 mM phosphate buffer (pH 7.4).
Different from RBC with 3mM tBHP alone (*** P< 0.005; **** P< 0.001) RBC suspension (HCT 10 %) was incubated with 3 mM tBHP for 30 min at 37°C. If MCS was used in these experiment, RBC suspension (HCT 10 %) was preincubated with 1.5 mM MCS for 45 min at 37°C,then washed for three times and resuspended in PBS (pH7.4) at 10 % HCT. If DTT or GSH was used in these experiment, 5 mM DTT or 5 mM GSH was added prior to tBHP. The data represent mean ± SD of four experiments.

TABLE III The effects of various antioxidants on the oxidation induced by tBHP

Sample	*TBARS (μmol MDA/L RBC)*	*MetHb (%)*	*OxyHb (%)*	*Haemichrome (%)*
RBC + tBHP	33.82 ± 4.19	56.9 ± 6.3	27.1 ± 3.8	16.0 ± 2.6
RBC + BHT + tBHP	10.91 ± 1.48****	52.8 ± 10.5	30.2 ± 6.1	17.0 ± 3.3
Lysates + BHT + tBHP	11.38 ± 1.87****	54.3 ± 8.9	27.8 ± 4.7	17.9 ± 4.2
RBC + ascorbate + tBHP	59.42 ± 8.31****	44.1 ± 9.5*	40.2 ± 7.6**	15.7 ± 1.1
Lysates + ascorbate + tBHP	81.25 ± 7.56****	24.2 ± 6.7****	75.1 ± 5.9****	0.6 ± 0.2****
RBC + DFO + tBHP	16.12 ± 2.86****	57.2 ± 11.3	25.4 ± 4.2	17.4 ± 2.2 2
Lysates + DFO + tBHP	17.94 ± 2.28****	23.7 ± 5.1***	62.4 ± 6.7****	5.6 ± 1.1****
Lysates + DFO + ascorbate + tBHP	35.12 ± 4.52	23.7 ± 3.6****	76.3 ± 5.8****	0.1 ± 0.1****
RBC + α-tocopherol + tBHP	18.61 ± 3.02****	58.9 ± 7.9	28.3 ± 3.2	12.8 ± 2.1
Lysates + α- tocopherol + tBHP	19.82 ± 3.25****	55.0 ± 5.8	30.9 ± 2.8	14.1± 2.3

Note. RBC; normal RBC.
Lysates; see Table II.
Different from RBC with 3mM tBHP alone (* P<0.05; ** P<0.01; *** P< 0.005; **** P< 0.001) RBC suspension (HCT 10 %) was incubated with 3 mM tBHP for 30 min at 37°C. If antioxidants were used in these experiment, 100 μM BHT, 5 mM ascorbate, 5mM α- tocopherol and 5mM DFO was added prior to tBHP in these systems. The data represent mean ± SD of four separate experiments.

Effects of Other Antioxidants on Oxidation Induced by tBHP

The effects of four antioxidants, butylated hydroxytoluene (BHT), ascorbate, α-tocopherol and desferrioxamine (DFO) were tested in two systems: a) intact normal RBC and b) lysates prepared from the same RBC. As shown in Table III, BHT was able to strongly inhibit the formation of TBARS but had virtually no effect on the oxidation of haemoglobin both in intact RBC and in lysates. BHT did not prevent the degradation and aggregation of membrane proteins (Fig. 6, Lane 3, 4).

Ascorbate provided partial protection against the oxidation of haemoglobin in intact normal RBC. After treatment of intact RBC with ascorbate, 44 % of OxyHb remained compared with 27 % of OxyHb in the presence of tBHP alone ($P<0.01$) (Table III). Ascorbate did not prevent the formation of haemichrome or the degradation and aggregation of membrane proteins in intact RBC (Table III and Fig. 6, lane 5). By contrast, the addition of ascorbate to the lysates provided significant protection against the oxidation of Hb (Table III). About 75 % of OxyHb remained and hardly any haemichrome was produced ($P<0.001$). Ascorbate almost completely prevented the degradation and aggregation of membrane proteins (Fig. 6, lane 6). Interestingly, however, ascorbate increased the formation of TBARS both in intact RBC and lysates (Table III).

DFO, an iron chelator, was partially effective in inhibiting the formation of TBARS but failed to prevent the oxidation of haemoglobin and the degradation and aggregation of membrane proteins induced by tBHP in intact RBC (Table III and Fig. 6, Lane 7). By contrast, DFO provided partial protection against the oxidation of haemoglobin and the degradation and aggregation of membrane proteins in lysates (Table III and Fig. 6, Lane 8). In the presence of 3 mM tBHP and 5mM DFO, the content of haemichrome was lower than that in the presence of tBHP alone ($P<0.001$) (Table III).Compared with tBHP incubation alone, the spectrins and band 3 only slightly decreased although the appearances of LMWP and HMWP were observed in the presence of tBHP and DFO (Fig. 6, Lane 8). We noticed that the formation of TBARS was significantly decreased by about 50 % and the degradation and aggregation of membrane proteins was prevented when lysates were incubated with ascorbate plus DFO (Table III, Fig. 6, Lane 9).

α-tocopherol was partially effective in preventing the formation of TBARS rather than the oxidation of haemoglobin induced by tBHP both in intact RBC and lysates (Table III). α-tocopherol partially prevented the degradation and aggregation of membrane proteins in intact RBC although formation of LMWP and HMWP still occurred in the presence of tBHP and α-tocopherol, (Fig. 6. Lane 10). α-tocopherol was less effective in protecting membrane proteins against the oxidation induced by tBHP in lysates (Fig. 6. Lane 11).

DISCUSSION

Erythrocyte GSH has been generally considered to provide protection against oxidative damage [2–5]. Our studies on the effects of tBHP on three types of RBC (RBC from high-GSH, low-GSH sheep and GSH-depleted RBC) showed that GSH significantly retards the process of oxidation induced by tBHP. Figs. 2 and 3 show that there is a significant lag in the appearance of all tBHP induced oxidation products in the early (0–10 min) time window (corresponding to the persistence of GSH) in the RBC from high-GSH sheep. These results indicate that loss of GSH is the first step in the tBHP mediated oxidation of RBC. After loss of GSH, oxidation of Hb resulted in a decrease in OxyHb and an increase in MetHb and haemichrome. Although oxidation induced by tBHP resulted in total GSH depletion within 10 min in the three types of RBC, effects of tBHP on the oxidation of Hb and the forma-

tion of TBARS were different. The levels of MetHb and TBARS in high-GSH RBC were significantly lower than those in the two other RBC after 5 min incubation. The content of OxyHb was higher and that of haemichrome in high-GSH RBC was lower compared with the other two RBC after 10 min incubation. The degradation and aggregation of membrane proteins did not occur within the first 10 min incubation in high-GSH RBC. These results clearly show that GSH is an important factor in protecting RBC against oxidative stress.

It has been reported that DTT can prevent lipid peroxidation, the inhibition of Ca^{2+} pump ATPase and the loss of OxyHb and GSH[14]. In our studies, DTT not only effectively inhibited the formation of TBARS and loss of OxyHb, but also prevented the degradation and aggregation of membrane proteins in both high-GSH and low-GSH RBC (Table I and Fig. 4, Lanes 8, 9). However, DTT did not prevent the formation of TBARS, the oxidation of haemoglobin and the degradation and aggregation of membrane proteins in GSH-depleted RBCs (Table I and Fig. 4, Lane 10). GSH-depleted RBC were made by incubating high-GSH RBC with 3 mM CDNB for 30 min. CDNB consumes GSH within RBC by forming an irreversible adduct, 2,4-dinitrophenol-S-glutathione (DNP-SG) while it does not affect GSH reductase (GR)or GSHPx activities [19]. It has been shown that DTT does not interfere with the measurement of DNP-SG efflux [27]. DTT is a membrane permeable agent [28,29]. We found that there were no differences between intact GSH-depleted RBC and lysates incubated with tBHP (data not shown). DTT could not be utilized by GSHPx as a substrate. The effect of DTT may be due to replenishing GSH from GSSG in the process of oxidation since DTT has been shown to maintain GSH levels even above that of control[30]. We observed that DTT did not prevent either the oxidation of Hb or the degradation and aggregation of membrane proteins in the presence of MCS, a potent inhibitor of GSHPx (TableII and Fig. 5, Lane 3). This is in agreement with the observation by Rohn et al that DTT does not protect RBC against the formation of MetHb and inhibition of Ca^{2+}-pump ATPase in the presence of MCS[14]. The protection of DTT against oxidative damage caused by tBHP is clearly a GSH-dependent mechanism. Our results are consistent with the hypothesis that the action of DTT is coupled directly to the GSH/GSHPx system [14].

tBHP itself is a substrate of GSHPx, the enzyme largely responsible for the oxidation of GSH to GSSG. To gain further insight on the GSH/GSHPx system, we tested the effect of exogenous GSH on this system. In intact RBC, the addition of GSH did not prevent the formation of TBARS, the oxidation of Hb and the degradation and aggregation of membrane proteins (Table II and Fig. 5, Lane 4). By contrast, in RBC lysates, GSH not only conferred effective protection on Hb and membrane proteins against oxidative damage induced by tBHP but also prevented the formation of TBARS (Table II and Fig. 5, Lane 5). It has been shown that RBC have no transport system for GSH[31]. tBHP is able to penetrate the lipid bilayer and oxidize intracellular constituents[14]. GSH added to the exterior of the normal RBC may not be utilized by GSHPx. Although it was previously reported that GSH itself binds to haemin using its thiol at physiological pH [32], here we show that the addition of GSH to the lysates preincubated with MCS did not inhibit the formation of TBARS, the oxidation of Hb and the degradation and aggregation of membrane proteins (Table II, Fig. 5, lane 6). Taken together, these data show that the protection conferred by GSH on RBCs against tBHP is mediated through the role of GSH as a substrate for GSHPx. Both GSH and GSHPx play critical roles in the system of GSH/GSHPx. If there is either insufficient GSH or GSHPx is inhibited, the enzymatic system becomes ineffective during oxidation induced by tBHP. If enough GSH is provided, GSHPx protects the RBC against oxidative damage by tBHP. The mechanism by which this enzymatic system provides protec-

tion against the oxidation induced by tBHP is through GSHPx mediated tBHP metabolism. As long as the GSH/GSHPx system is fully operational, it mitigates the formation of TBARS and the oxidation of Hb as well as the degradation and aggregation of membrane proteins in the presence of tBHP (Table II and Fig. 5, lane 5).

Since tBHP has little effect on the membrane ATPases and membrane proteins if it is incubated with white membrane ghosts which do not trigger free radical production [33], free radicals produced from tBHP must play a key role in the oxidative process. There are two types of radical, alkoxyl radical (t-BuO·) and alkyl peroxyl radicals (t-BuOO·) produced by the reaction of tBHP and Hb [34,35]. Recent studies indicate that t-BuO· is the initial radical and t-BuOO· is the secondary product produced by scission of t-BuO· and subsequent reaction [36,37].

It is not clear which one of the aforementioned two radicals is more important in the degradation of RBC membrane proteins induced by tBHP. Ascorbate is an excellent scavenger either of t-BuO·[13] or of t-BuOO· [38] so it is reasonable to assume that it may completely protect RBC membranes against the attack by these radicals in our lysate experiment (Fig 6, Lane 6). Ascorbate does not however provide effective protection in intact RBC (Fig 6, Lane 5). The difference may be explained on the basis that reduced ascorbate is transported across cell membranes with a much lower efficiency [39] (Table III). Partial protection of Hb by ascorbate may be mediated by its ability to reduce MetHb [40] and ferryl Hb[41]. In our study, increase in TBARS and protection of the degradation and aggregation of membrane proteins by ascorbate indicate that ascorbate may act both as an antioxidant as well as a prooxidant in this process. Increase in TBARS by ascorbate was probably related to iron since DFO (iron chelator) counteracted the effect of ascorbate (Table III).

Although DFO is primarily used as an iron chelator, it has been shown to scavenge both t-BuO· and t-BuOO· in a model system utilizing tBHP and MetHb independently of its iron chelating property [42]. Partial protection of membrane proteins in lysates against tBHP by DFO (Fig. 6, Lane 8) may be due to the latter ability. Like ascorbate, the differential effects of DFO between intact RBC and lysates on the oxidative induced by tBHP may be due to its very slow penetration into cells [43] (Fig. 6, Lanes 7, 8)

It has been shown that α-tocopherol is a potent scavenger of t-BuOO·[38], although other reports indicate that α-tocopherol can also act as a scavenger of t-BuO·[44,45]. Here we show that α-tocopherol partially prevents the degradation and aggregation of membrane proteins (Fig. 6, lanes 10,11). BHT on the other hand is a very poor scavenger of t-BuO·[13] but a moderate scavenger of t-BuOO·[38,46]. Here we show BHT to be ineffective in preventing the degradation and aggregation of membrane proteins (Fig 6, Lanes 3,4). According to our results, the protection or otherwise of RBC against the degradation and aggregation of membrane proteins induced by tBHP afforded by the four added antioxidants seems to depend on their differential ability to scavenge the radicals, t-BuO· and t-BuOO·. However, more experimental data are required to resolve this important question.

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