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t-Butyl hydroperoxide-induced perturbations of human erythrocytes as a model for oxidant stress

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Erythrocytes were incubated with *t*-butyl hydroperoxide in the presence and absence of hemoglobin as a model system for oxidative stress and the alterations in the structure and integrity of the membranes were investigated. The results showed that in the presence of hemoglobin a significant modification in the membrane surface charge was induced but no such alteration was observed in peroxidised hemoglobin-free membranes. As increased hemoglobin oxidation occurred in the erythrocytes, membrane lipid peroxidation diminished, suggesting a protective role for methemoglobin in *t*-butyl hydroperoxide-induced lipid peroxidation. Electrophoresis on polyacrylamide gels showed modification of the cytoplasmic protein region but no high molecular weight aggregates formed at the concentrations of the hydroperoxide used in this work. The results suggest that the *t*-butyl hydroperoxide/normal erythrocyte system seems to be an instructive model for membrane perturbations characteristic of oxidative disorders.

Introduction

Membrane lipid peroxidation is associated with shortened red survival in a number of hemolytic states due to either congenital or acquired defects, such as oxidant drug-induced hemolysis [1] and β-thalassemia major [2], as well as in the aging of red cells [3].

Red cell damage by oxidant stress is generally thought to be the end result of two processes. The oxidation of hemoglobin occurs followed by the denaturation of methemoglobin to hemichrome and the subsequent binding of denatured hemicrome to the membrane [4] leading to cell lysis [5]. The polyunsaturated fatty acid sidechains of the membrane lipids are susceptible to attack by oxidising radicals with the formation of lipid hydroperoxides. The latter eventually decompose to a variety of end-products including malonyldialdehyde [6] which is not a normal constituent of erythrocytes, and therefore its production provides a measure of the susceptibility of membrane lipids to peroxidation.

Our earlier work [7–11] has attempted to establish the basic modifications in erythrocyte membrane structure and interactions which occur when normal erythrocytes are exposed in vitro to autoxidisable agents which can react with a variety of active species. Trotta et al. [12–14] have investigated the mechanism of *t*-butyl hydroperoxide-induced hemoglobin oxidation and lipid peroxidation in relation to hydroperoxide consumption in

Abbreviation: ANS, 1-anilino-8-naphthalene sulfonate (magnesium salt).

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order to determine the relative roles of the initiation, propagation and termination reactions.

The aim of the present study was to determine the alterations in the structure and integrity of the membrane after exposure of erythrocytes and hemoglobin-free membranes to *t*-butyl hydroperoxide as a model system for oxidant stress. The results suggest: (i) a potential role for hemoglobin in modifying the membrane surface charge when the erythrocyte is under oxidative stress; (ii) that methemoglobin has a protective role in *t*-butyl hydroperoxide-induced peroxidation of membrane lipids; (iii) that the *t*-butyl hydroperoxide/normal human erythrocyte system is an instructive model for studying membrane perturbations in hemolytic disorders involving oxidative hemoglobin defects, such as β-thalassemia major.

Methods

Fresh blood from normal donors was taken into heparin and used immediately. The red cells were separated, washed three times in iso-osmotic Tris-NaCl buffer (pH 7.4) and incubated at a 5% hematocrit in *t*-butyl hydroperoxide of the appropriate concentration in iso-osmotic Tris buffer at 25°C and 37° for the appropriate time interval in a shaking water bath. Control cells were similarly incubated in the absence of the hydroperoxide. After incubation the cells were washed twice.

Samples were fixed at a 5% concentration in glutaraldehyde in cacodylate buffer, dehydrated and critical-point dried. Samples for scanning electron microscopy were prepared using carbon and gold in an Ion Tech Saddle field using an ion source sputter coater. Micrographs were taken on a Philips 501 scanning electronmicroscope. Further samples of washed erythrocytes were removed for total phospholipid [15] and cholesterol assays. Methemoglobin levels were measured using the standard method of cyanide treatment and observing the decrease in absorbance at 620 nm [16]. Total hemoglobin was estimated as cyanomethemoglobin using Drabkin's reagent [17].

Membranes were prepared by hypotonic haemolysis using standard procedures [18] but applying Tris buffers and ghosts were finally suspended in iso-osmotic Tris-HCl buffer in saline (pH 7.4). Control hemoglobin-free membranes were treated with 1 mM *t*-butyl hydroperoxide at 37°C for various time periods. The membrane protein concentration was determined by the method of Lowry et al. [19] using bovine serum albumin (Sigma) as standard. Free thiol sidechains on the membrane proteins were determined spectrophotometrically at 412 nm using the Ellman method [20] involving 5,5′-dithiobis(2-nitrobenzoic acid) (Sigma) as the colour reagent and using glutathione (reduced form) (Sigma) as standard.

Membrane lipid peroxidation was assayed using the following methods:

(i) A modification of the method of Kumar et al. [21] involving the thiobarbituric acid reaction of free malonyldialdehyde: one volume of membranes prepared from the control and treated cells was mixed with one volume of 10% trichloroacetic acid and one volume of 0.75% thiobarbituric in 0.1 M hydrochloric acid. The mixture was heated for 20 min at 90–95°C followed by centrifugation for 10 min at $5000 \times g$. The pink chromophore was assayed spectrometrically at 532 nm.

(ii) The spectrofluorimetric assay [22] for malonyldialdehyde bound via Schiff's base formation to appropriate groups on the phospholipid sidechains: lipids extracted [15] from the control and treated samples were suspended in chloroform (spectroscopic grade) and the fluorescence characteristics of the cross-linked lipids were analysed on a Perkin-Elmer MPF 44B fluorescence spectrophotometer using an excitation wavelength of 350 nm and emission spectra were recorded between 400 and 500 nm. The instrument was standardised using quinine sulphate (100 μg/ml 0.05 M sulphuric acid);

(iii) Electrophoresis on polyacrylamide gels for investigating malonyldialdehyde bound to protein sidechains to form high molecular weight aggregates. Electrophoresis on polyacrylamide gels was performed following standard procedures [23] using 5% acrylamide gels. Proteins were visualized after staining with Kenacid blue (BDH).

The relative microviscosity of the membrane lipids was measured after incorporation of the apolar fluidity probe 1,6-diphenyl-1,3,5-hexatriene (Aldrich) as described previously [24] using a probe-lipid ratio of 0.007. Glan prism polarisers were fitted to the Aminco-Bowman spectrofluorometer for polarisation of fluorescence (p) de-

terminations. Excitation was from a vertically polarised light at 365 nm and the polarised emission was observed through an analyser oriented parallel (I_{vv}) and perpendicular (I_{vh}) to the direction of the excitation light. Fluorescence polarisation is given by the equation:

$$P = \frac{I_{vv} - T \cdot I_{vh}}{I_{vv} + T \cdot I_{vh}}$$

where T is the ratio I_{hv}/I_{vh} when the excitation beam is horizontally polarised and the emission observed through analysers oriented perpendicularly and parallel, respectively.

The observed polarisation, p, the average response under specific conditions is transposed into fluorescence anisotropy, r, $(r = 2p/(3-p))$ and then converted into the relative microviscosity parameter $((r_0/r)-1)^{-1}$, which is directly proportional to the microviscosity, where r_0 is the limiting anisotropy for this probe, 0.362 [25].

Alterations in the charge at the membrane surface were monitored using the amphipathic fluorescent probe 1-anilino-8-naphthalene sulfonate (ANS, magnesium salt, Eastman). Membrane samples contained 53.3 μg membrane protein/ml and 10 μM ANS, and measurements were performed as described previously [26]. The extrinsic fluorescence of ANS was excited at 350 nm and the emission observed at 460 nm.

Results

Exposure of erythrocytes to 0.25 mM *t*-butyl hydroperoxide for 60 min at 37°C induced gross morphological changes to the echinocyte conformation consisting of spherical cells with spicules distributed over their surfaces (Fig. 1).

Incubation of cells with the hydroperoxide resulted in both lipid peroxidation and hemoglobin oxidation. Progressively increasing levels of thiobarbituric acid-reactive secondary breakdown products of membrane lipid peroxidation with time of incubation (Table I) up to 60 min were produced. At the elevated concentrations of the hydroperoxide or with the prolonged incubation time a diminished degree of peroxidation in terms of the production of free malonyldialdehyde was observed. This apparent reduction was not accounted for by the increased binding of malonyldialdehyde via Schiff's base formation to the membrane lipid components (Fig. 2) since the relative fluorescence intensity per μg phospholipid of the chromolipids was almost the same under conditions of incubation with the hydroperoxide involving 0.25 mM (60 min) as with 0.4 mM (90 min). The conditions under which the apparent resistance to membrane lipid peroxidation developed were those at which the accumulation of methemoglobin was the greatest.

Electrophoresis on polyacrylamide gels (Fig. 3) demonstrated no new protein aggregates at the high molecular weight end nor throughout the lower molecular weight region. An interesting modification of the protein profile of the peroxidised erythrocytes, however, was the disappearance of the band 2.1-ankyrin component of the membrane cytoskeletal network with a con-

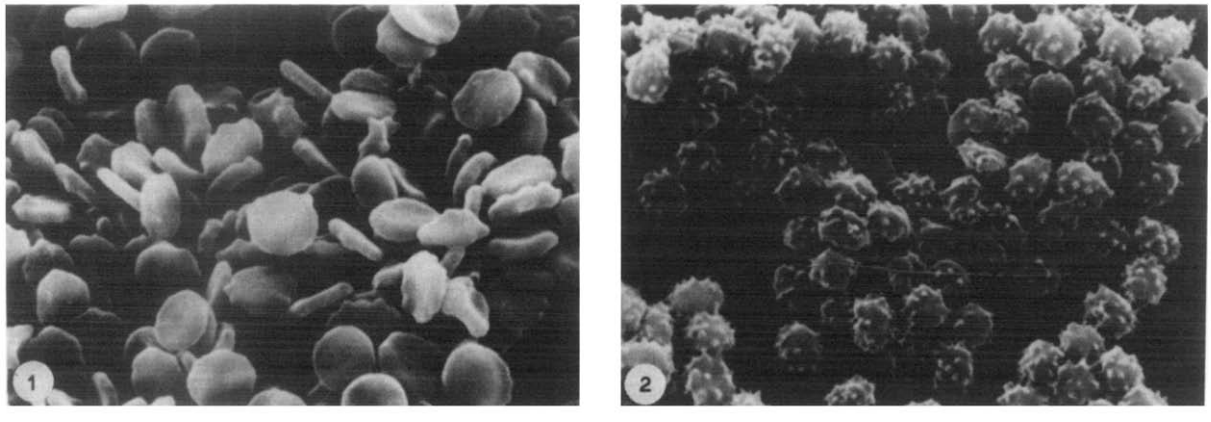

Fig. 1. Scanning electron micrographs of control human erythrocytes (1) and *t*-butyl hydroperoxide-treated erythrocytes, 0.25 mM, 60 min, 37°C (2), (×1870).

TABLE I

ALTERED MEMBRANE CHARACTERISTICS OF HUMAN ERYTHROCYTE MEMBRANES INDUCED BY *t*-BUTYL HYDROPEROXIDE

The number in brackets represents the number of separate experiments each of which was performed in triplicate.

Conditions of incubation	Peroxidation (A_{532}/mg protein)	Membrane protein free thiol level (nmol/mg protein)	Relative microviscosity $((r_0/r)-1)^{-1}$	% Decrease in ANS fluorescence	% Methemoglobin
0.0 mM, 90 min	0.030 ± 0.004 (9)	78 ± 4 (9)	1.58 ± 0.08 (9)	0	0
0.25 mM, 15 min	0.089 ± 0.006 (4)	–	–	–	–
0.25 mM, 60 min	0.103 ± 0.006 (8)	46 ± 5 (8)	–	25 (4)	30 (3)
0.25 mM, 90 min	0.040 ± 0.004 (4)	46 ± 5 (4)	2.00 ± 0.10 (4)	25 (4)	–
0.40 mM, 90 min	0.040 ± 0.004 (8)	–	2.00 ± 0.12 (8)	25 (4)	46 (3)

comitant increase in the band 2.3 component. A similar change in the ankyrin bands was also observed in the thalassaemic membranes from some patients with β-thalassemia major (Fig. 3).

Measurements of the free thiol sidechain content (Table I) of the membrane proteins in the hydroperoxide-treated erythrocytes showed a 40% decrease in free -SH groups but there was no indication of mutually crosslinked membrane proteins via disulfide interactions from the electrophoresis studies. This implies that either hemoglobin-protein disulfide crosslinks may be involved, or more specifically mixed disulfide linkages with denatured hemoglobin [27]. It is also conceivable that decomposition products from the oxidative effects of the polyunsaturated fatty acid chains of the membrane lipids may bind to free thiol groups under these conditions.

Incorporation of the fluidity probe, diphenylhexatriene, into the membranes prepared from the treated and control erythrocytes (Table I) showed that the hydrophobic lipid region of the membrane became less fluid on peroxidation of the polyunsaturated fatty acid sidechains although the overall cholesterol/phospholipid ratio was unaffected.

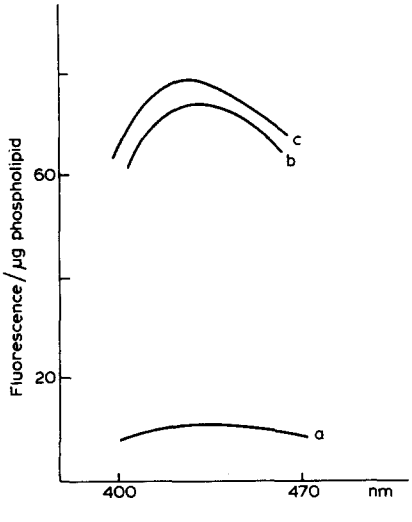

Fig. 2. Chromolipid formation in peroxidised erythrocytes: (a) control red cells; (b) 0.25 mM, 60 min, 37°C treatment; (c) 0.40 mM, 90 min, 37°C treatment.

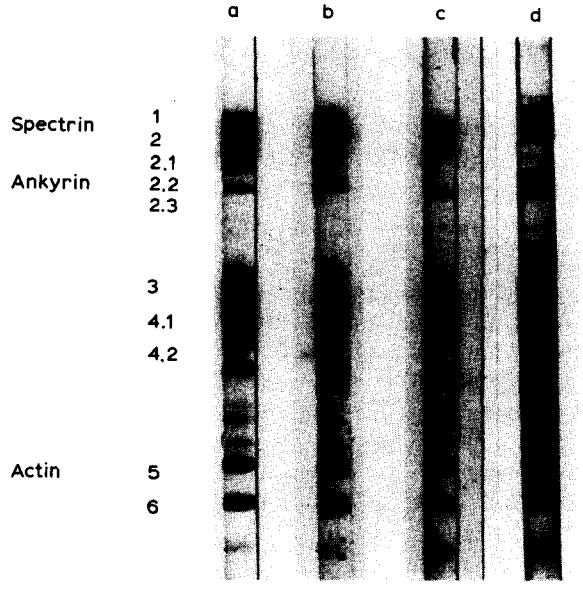

Fig. 3. Polyacrylamide gel electrophoresis of erythrocyte membrane proteins: (a) control membranes; (b) *t*-butyl hydroperoxide-treated 0.25 mM, 60 min, 37°C; (c) *t*-butyl hydroperoxide-treated 0.40 mM, 90 min, 37°C; (d) thalassemic membranes, untreated.

To monitor changes in charge or polarity at the membrane surface the fluorescent probe 1-anilino-8-naphthalene sulfonate (ANS) was applied. Erythrocyte membranes prepared from *t*-butyl hydroperoxide-treated erythrocytes showed a pronounced decrease in the ANS fluorescence intensity (Table I) with no shift in the emission maximum wavelength (460 nm). Hence, this is not simply a polarity effect [28] altering the accessibility of the probe to the aqueous environment but rather is indicative of an increased negative charge at the membrane surface.

In order to assess the contribution of hemoglobin towards the membrane changes observed when the erythrocytes are exposed to *t*-butyl hydroperoxide, experiments were carried out on hemoglobin-free membranes but at the concentration of 1 mM hydroperoxide. As shown in Fig. 4 the result was (i) a progressively increased production of free malonyldialdehyde with time of incubation; (ii) a progressive oxidation of free thiol sidechains of the membrane proteins; (iii) a decreased average bulk lipid fluidity, all of which were similar to the overall alterations observed in treated intact erythrocytes.

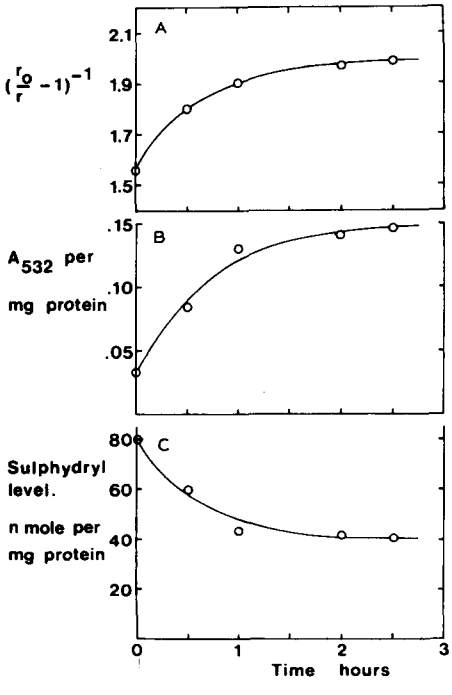

Fig. 4. Membrane alterations in hemoglobin-free erythrocyte membranes treated with 1 mM *t*-butyl hydroperoxide: relative microviscosity, 37°C (A); peroxidation levels (B); free thiol levels of the membrane protein sidechains (C).

However, no modification in the charge at the membrane surface was observed in the membranes peroxidised in the absence of hemoglobin.

Discussion

Our model system has been applied to investigate the modifications in the structural integrity of erythrocyte membranes under the influence of *t*-butyl hydroperoxide, and the role of haemoglobin oxidation and its subsequent interactions with the membrane.

The membrane perturbations induced by exposing erythrocytes and hemoglobin-free membranes to *t*-butyl hydroperoxide result partly from the decomposition of lipid hydroperoxides to malonyldialdehyde. Peroxidation occurs both in the erythrocyte system by heme-promoted decomposition of the *t*-butyl hydroperoxide [14] and in the hemoglobin-free membrane system. The direct role of membrane-bound hemoglobin in increasing the rigidity of the membrane lipids is probably less significant than the formation of the phospholipid adducts by cross-linking of the membrane lipids involving malonyldialdehyde since the peroxidation of the hemoglobin-free membrane lipids has a similar rigidifying effect on the lipid region.

The response of the incorporation of the polarity probe, ANS, in peroxidised erythrocytes is indicative of an increased negative charge at the membrane surface, possibly resulting from changes in the packing and orientation of the phospholipids and is not merely a polarity effect on the environment of the probe. This observation is supported by phase partition studies [10]. In comparison the lack of any effect on ANS incorporated into membranes peroxidised in the absence of hemoglobin suggests that hemoglobin appears to have a role in catalysing the action of *t*-butyl hydroperoxide on those membrane components contributing towards the overall charge at the membrane surface. Membrane lipid asymmetry, a surface property of potential importance to the magnitude and distribution of the membrane surface charge, may be involved here. This possibility is currently under investigation. The major

contribution to the charge at the red cell surface arises from the extracellularly exposed carbohydrate portions of the the glycoproteins and glycolipids, whose hydrophobic groups extend into the membrane lipid interior. It has been postulated that interactions between the transmembrane glycoproteins and the protein constituents of the membrane skeleton at the cytoplasmic surface of the membrane may keep the glycoproteins uniformly distributed in normal cells [29]. The apparent loss of the band 2.1 component of the cytoskeletal protein, ankyrin, when erythrocytes are treated with *t*-butyl hydroperoxide observed in this study may be indicative of an altered distribution of the glycoproteins under these conditions.

Increased oxidation of hemoglobin to methemoglobin was not parallelled by increased lipid peroxidation either in terms of free malonyldialdehyde or malonyldialdehyde bound to the membrane lipids, but as methemoglobin production progressed the total malonyldialdehyde production declined overall. The observation implies that methemoglobin may have a protective role for the polyunsaturated fatty acid sidechains against lipid peroxidation. This finding is consistent with the recent work of Trotta et al. [14] who have postulated that red cells exposed to *t*-butyl hydroperoxide represent extremes of oxidative damage, namely, membrane peroxidation and hemoglobin degradation, such that the conditions that cause increased hemoglobin degradation have a sparing effect on the membrane lipids. It is possible that methemoglobin is acting as a scavenger of radical intermediates of propagating fatty acid chains of membrane lipids or of radical intermediates related to the mechanism of action of *t*-butyl hydroperoxide in oxidative processes, e.g. the *t*-butoxy radical.

The failure to observe irreducible high molecular weight protein aggregates in the membranes of peroxidised erythrocytes in our studies demonstrates the lack of interaction of malonyldialdehyde with the membrane proteins at the concentrations of *t*-butyl hydroxyperoxide used. Other workers have shown high molecular weight crosslinked protein aggregates but their oxidative conditions involved higher concentrations of the hydroperoxide [30].

Evidence for the potential of malonyldialdehyde reactivity with membrane proteins has been presented in the work of Jain et al. involving the treatment of erythrocytes in vitro with exogenously added malonyldialdehyde on the one hand [3], and malonyldialdehyde formed endogenously during oxidative breakdown of phospholipids in erythrocyte membranes from iron-deficient rats on the other hand [31]. These experiments produced crosslinked aggregates of protein involving spectrin as well as Schiff's base interactions involving the aminophospholipids. Flynn et al. [32] have clearly shown convincing evidence for aggregated membrane proteins in the oxidative disorder Hemoglobin Köln disease. Furthermore, Kahane et al. [2] suggest that thalassemic membranes also show oxidative protein crosslinks which were not reversed after vitamin E administration to the patients. These findings are contradicted by our electrophoretic data which have persistently failed to show high molecular weight protein complexes in freshly prepared thalassemic membranes.

It can be concluded from this study that *t*-butyl hydroperoxide/normal human erythrocyte model system seems to be a useful one for membrane perturbations characteristic of oxidative disorders. For example, all the membrane abnormalities observed in this model system have been demonstrated in studies on the erythrocyte membranes from patients with β-thalassemia major [33,34]. The important observations which correlate are our measurements on the decreased lipid fluidity, decreased free thiol sidechains on the membrane proteins, increased negative charge at the membrane surface [33], the disappearance of the band 2.1-ankyrin component of the membrane proteins. This latter observation is not a generalised one for all patients and may be related to the proportion of thalassemic cells, rather than transfused cells, in the circulation, in other words, the time lapse since transfusion.

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