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Prehemolytic effects of hydrogen peroxide and *t*-butylhydroperoxide on selected red cell properties

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To provide further understanding of how oxidative damage affects red cell membrane function, the effects of low levels of two different types of oxidants on selected red cell properties have been studied. Hydrogen peroxide (H_2O_2), an example of a water soluble oxidant, and *t*-butylhydroperoxide (tBHP), a hydrophobic hydroperoxide, were compared with respect to their effects on membrane permeability, membrane mechanical properties and binding of autologous serum antibodies to the cell surface. Whereas H_2O_2 treatment resulted in a dose-dependent increase in membrane permeability to potassium that was evident after one hour of oxidant exposure, cells treated with tBHP at doses up to 5 μmol / ml cells showed no immediate change in cation permeability. H_2O_2 also caused a marked decrease in membrane deformability, whereas tBHP-treated cells showed minimal loss of deformability. However, tBHP treatment did result in a dose-dependent increase in the susceptibility of the membrane to fragmentation under high shear stress. With exclusion of treated samples that bound excess rabbit anti-spectrin antibody, indicating exposure of intracellular components, neither agent promoted the binding of autologous serum antibody in amounts comparable to that found *in vivo* on high density or some pathologic red cells. Taken together, the results suggest that tBHP and H_2O_2 cause damage to human red cells by distinct oxidative mechanisms which do not lead directly to substantive generation of binding sites for autologous serum antibodies.

Introduction

Currently, a great deal of interest in the area of red cell senescence is focused on the role of cumulative oxidative damage in generating immunologically recognizable alterations in the cell surface [1–3] that may promote binding of autologous antibodies and clearance of senescent cells from the circulation [2,4–6]. In addition, for a variety of disorders involving hemoglobin abnormalities, it has been proposed that enhanced oxidative damage plays a role in accelerating red cell destruction [7]. To further explore the potential for oxidative processes to produce changes that may accompany red cell senescence or that are characteristic of hemoglobinopathic red cells, we have studied the effects of two different types of oxidants on selected red cell properties and on the capacity of the cell to bind autologous serum antibodies.

As model oxidants, we used hydrogen peroxide (H_2O_2), a representative water-soluble peroxide, and *t*-butylhydroperoxide (tBHP), a hydrophobic hydroperoxide. To obtain insights into how damage by these two types of oxidant might relate to the process of red cell destruction, we determined the effects of the two agents on red cell properties that have been shown to have major effects on red cell survival: membrane cation permeability and cell water content, membrane deformability and mechanical stability, and the binding of autologous serum antibodies.

Extensive oxidative damage to red cells can lead to hemolysis. Because hemolysis itself produces changes in membrane permeability, cell deformability and binding of autologous serum antibody, it is not possible to study the effect of oxidant damage on these properties when hemolysis occurs. Therefore, we have focused

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our studies on cells treated at low oxidant levels that do not induce hemolysis and have confined our attention to prehemolytic changes that result from oxidative stress.

Methods

Blood was drawn from normal adult volunteers using an informed consent protocol approved by the Committees on Human Research at the University of California, San Francisco and at San Francisco State University. Red cells were obtained from samples drawn into acid-citrate-dextrose anticoagulant and serum from samples without anticoagulant. The serum was heated to 56°C for 40 min to inactivate complement. Before treatment with oxidants, red cells were washed three times in phosphate-buffered saline (PBS: 1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, 137 mM NaCl (pH 7.4)), and the buffy coat aspirated each time.

Treatment with oxidants. Washed red cells were incubated at 20% hematocrit (hct) in PBS containing H_2O_2 (0.5 to 3.0 mM) or tBHP (0.5 to 1.5 mM). This range was chosen to give the maximum treatment possible without inducing hemolysis. To obtain uniform exposure of the cells to oxidants, the untreated suspensions were first centrifuged, and the supernatant removed. The oxidant was added to the supernatant, which was then recombined with the packed cells. Sodium azide (3 mM) was added to the H_2O_2-treated samples to prevent rapid destruction of the peroxide by catalase. As in studies by most other investigators, tBHP treatments were performed in the absence of azide. Determination of cation fluxes after tBHP treatment with and without azide showed that azide had no modifying influence on this measure of membrane damage over the range of tBHP doses used in these experiments. Its presence at much higher levels of tBHP treatment has been reported to suppress precipitation of non-intact Hb and to enhance oxidation-induced increase in Cl^- permeability [8]. However, those studies involved a much greater extent of Hb and lipid oxidation than ours and are thus not directly comparable. Samples were incubated at 37°C for one hour, and were then centrifuged and resuspended without oxidant at 10% hct in buffered saline containing glucose (BSKG: 1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, 134 mM NaCl, 5 mM KCl, and 10 mM glucose (pH 7.4)). For experiments involving prolonged incubation, 150 u/ml of penicillin and 0.15 mg/ml of streptomycin were added to inhibit bacterial growth. In some experiments, before treatment with the oxidants, paired samples were prepared and one of each pair was presaturated with carbon monoxide (CO) to block processes mediated by hemoglobin oxidation.

To verify that the small amounts of added tBHP were taken up by the cells, samples of suspension supernatants were taken at 5 and 60 min after initiation of 37°C incubation with tBHP. These were assayed for residual tBHP as described by Trotta et al. [9]. Samples of tBHP diluted into buffer samples without cells were assayed in parallel. It was found that the cells took up the vast majority of the added tBHP within the first 5 min of incubation.

To determine whether exposure to tBHP had resulted in measurable alkylation of membrane protein sulfhydryl groups, we determined binding of *N*-ethyl[^{14}C]maleimide ([^{14}C]NEM) to membrane proteins. Cells were first treated with 1 mM tBHP for 1 h at 37°C, and were then washed once and incubated overnight at 37°C in BSKG with penicillin/streptomycin. Resealed ghosts were prepared as for ektacytometric assay, and these were treated with 1.0 and 0.5 mM [^{14}C]NEM (specific activity 2.5 μCi/mmol) at 37°C for 30 min. Parallel samples treated with non-radioactive NEM were subjected to ektacytometric assay to determine whether membrane fragility had been altered. Radiolabeled samples were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) [10] and stained with Coomassie blue. The regions corresponding to spectrin, ankyrin, band 3 and band 5 were then cut out assayed for bound [^{14}C]NEM. Coomassie blue stain was eluted from duplicate samples and quantitated spectrophotometrically [10]. The ratio of radioactivity to eluted stain was used to determine whether tBHP treatment had resulted in any change in the binding of [^{14}C]NEM for any of these membrane components.

Measurement of lipid and hemoglobin oxidation. Oxidation of Hb to methemoglobin was detected by measuring the absorbance of well-oxygenated lysates at 540, 560, 570, 576 and 630 nm. We then used reported extinction coefficients for oxy, deoxy, carbomonoxy and methemoglobin at these wavelengths [11] to solve simultaneous linear equations for these species, employing the Eureka program (Borland, Inc, Scotts Valley, CA) and an Apple Macintosh II computer. Because there were data for more wavelengths than absorbing species, we performed calculations using all possible combinations of three (for oxygenated samples) or four (CO samples) wavelengths, with the stipulation that the data from 630 nm, which has the strongest contribution from methemoglobin, was always included. The values for methemoglobin concentrations obtained for each set of calculations were then averaged to provide the best estimate. As a measure of lipid peroxidation, the production of malonyldialdehyde (MDA) was measured via its reaction with thiobarbituric acid (TBA) [12]. As emphasized by Gilbert et al., this assay can be compromised by generation of interfering chromagenic substances. [13]. We therefore scanned the spectrum from 300 to 600 nm to detect such species. It was found that in the H_2O_2 samples, species were generated that

showed an absorbtion maximum around 465 nm, with a broad shoulder extending out to 532 nm, where the MDA-TBA complex absorbs maximally. Because the spectrum was somewhat different from that reported by Gilbert et al., it was not possible to apply the simple correction which they suggested. The substances producing this interference were not removed by washing the red cells before extraction and treatment with TBA, whereas the characteristic MDA/TBA spectrum in extracts from tBHP-treated cells was lost or markedly reduced by prior washing (data not shown). From this we concluded that very little of the absorbance at 532 nm in extracts from unwashed H_2O_2-treated cells was due to MDA generated by lipid peroxidation. Only the amount of MDA produced in tBHP-treated cells was calculated, and it was expressed in nanomoles of MDA per g of hemoglobin.

Cation flux measurements. To determine oxidant effects on cation permeability, Na^+ and K^+ fluxes were measured, using 10% cell suspensions in K^+-free PBS containing 0.1 mM ouabain to inhibit active cation transport [14]. Intracellular Na^+ and extracellular K^+ concentrations were measured at 30-min intervals over a 2 h period, using flame photometry. Intracelluar cations were determined on cell samples washed three times in isotonic Tris-buffered magnesium chloride solution (10 mM Tris-HCl, pH 7.4 at 0°C). Intracellular Na^+ concentrations were used to determine rates of Na^+ uptake, and extracellular K^+ concentrations for K^+ loss.

The presence of possible net changes in cation and cell water content was evaluated by centrifuging cell samples on 7-layer (1.087–1.114 g/ml) discontinuous gradients of arabinogalactan density gradient medium [15] (Larex-Lo, Consulting Associates, Inc, Tacoma, WA) and looking for differences in cell density distribution.

Measurement of cell and membrane deformability and membrane stability. To further evaluate possible changes in cellular hydration and in mechanical properties, we used an ektacytometer to measure whole cell deformability as a function of suspending medium osmolality [16], membrane deformability as a function of applied shear stress [17], and the rate of membrane fragmentation at high, constant shear stress [18]. The latter two assays were performed using resealed 'ghost' preparations, as originally described.

Binding of autologous serum antibodies. After treatment with oxidant, the cell samples were washed once and incubated overnight at 37°C in BSKG containing penicillin and streptomycin. They were then exposed to heat-inactivated autologous serum (10^8 cells in 0.5 ml) for 1 h at 37°C. After three washes in PBS, they were assayed for binding of autologous serum antibodies, using ^{125}I-Protein A as described elsewhere [19]. In order to exclude samples in which severe membrane damage had exposed intracellular binding sites for autologous antibody, parallel samples were incubated with affinity purified, rabbit anti-human spectrin (gift of Mary E. Rossi, University of California, San Francisco). Any samples that showed binding of Protein A after exposure to anti-spectrin that was higher than that observed in untreated cells exposed to autologous serum were considered to have a breach in membrane integrity, and the results from these samples were excluded. In a few experiments, the binding of Protein A to cells exposed to autologous serum was much higher than that to cells exposed to anti-spectrin. Because the untreated cells for the paired assays should bind equal quantities of Protein A, reflecting nonspecific binding and in situ IgG, it appeared that in these experiments, there may have been aggregated IgG in the serum that bound to the red cells. Therefore these experiments were also excluded. Specific activity of Protein A varied from experiment to experiment. Therefore, in analyzing the data, the counts per cell for treated cells were divided by the counts per cell for control, untreated cells, to determine whether treatment increased the amount of bound antibody. Counts ranged from 800 to 9500 cpm per sample of 10^8 cells.

Anti-hemoglobin immunoblots. For analysis of hemoglobin association with the membrane and membrane proteins, cells were hemolyzed and washed in hypotonic buffer (7 mM KCl, 5 mM Tris-HCl, 0.5 mM EGTA (pH 7.4)) until the supernatant was colorless. Membrane proteins were separated on polyacrylamide gels [20], and then transferred to nitrocellulose paper for immunoblotting [21] with rabbit anti-human globin (Sigma Chemicals, St. Louis, MO). Before use, the anti-globin was partially purified using human red cell hemolyzate on an Affigel column (Bio-Rad, Richmond, CA).

Analysis of results. Results of assays comparing treated cells with untreated control samples were analyzed, using a *t*-test for paired or unpaired samples, as appropriate. This was done using the Statview program on a Macintosh II computer.

Results

Cation permeability and cell density distribution

H_2O_2 and tBHP had disparate effects on red cell cation permeability under the conditions of these experiments. As shown in cation flux assays performed immediately after exposure to the oxidants (Table I), H_2O_2 caused a marked, dose-dependent increase in K^+ efflux, whereas tBHP had only minimal effect. The results of Na^+ influx measurements were less clear cut, because of the inherently lower reliability of measurements of relatively small changes in the intracellular Na^+ concentration. However, they were qualitatively

TABLE I

Effects of tBHP and H_2O_2 on K^+ efflux

First-order rate constants (h^{-1}) are given for cells treated with indicated oxidant concentrations. The values shown are means from four experiments for each oxidant, and the numbers in parentheses represent the S.E. Flux measurements were performed after a 1 h incubation of cells with the oxidant, and after the cells had been resuspended in fresh, K^+-free medium.

Oxidant		First-order rate constant of K^+ efflux			
tBHP	Concn.:	0	0.5 mM	0.75 mM	1.0 mM
		0.016 (0.001)	0.017 (0.002)	0.018 (0.001)	0.019 (0.001)
H_2O_2	Concn.:	0	0.5 mM	1.0 mM	1.5 mM
		0.018 (0.003)	0.020 (0.003)	0.030 (0.004)	0.041 (0.004)

similar, showing increased permeability in cells treated with 1.0 and 1.5 mM H_2O_2, but no change in cells treated with tBHP up to 1 mM concentration (data not shown). Analysis of red cell density distribution by centrifugation on arabinogalactan gradients showed only minor increases in the number of high-density cells in H_2O_2-treated samples by 2 h after the end of the 1 h treatment period. There was a suggestion of a minor reduction in the proportion of high-density cells in tBHP-treated samples (data not shown). However, as discussed below, results of the ektacytometric osmotic gradient analysis performed after overnight incubation indicated that at that time there was no major shift in the hydration status of the whole cell population.

Hemoglobin and lipid oxidation. In these experiments, Hb oxidation was more extensive during treatment with H_2O_2 than with tBHP and increased with increasing oxidant concentration (Table II). As expected, formation of methemoglobin in the presence of H_2O_2 was strongly inhibited by pretreatment of the cells with carbon monoxide; the methemoglobin formation in the presence of the higher concentration of tBHP was slightly reduced by pretreatment with carbon monoxide.

The increased Hb oxidation in H_2O_2-treated cells was accompanied by greatly increased adherence of Hb to the membrane. This was indicated by immunoblots prepared from well-washed ghosts, in which anti-globin was used to detect the globin that was still associated with the membrane. As shown in Fig. 1, H_2O_2-treated ghosts retained much more globin than tBHP-treated ghosts. Strong bands were seen at positions corresponding to apparent molecular masses of approx. 30–35 and 49–53 kDa. They are essentially the same as those bands observed in membrane-free hemolyzate cross-linked with dimethyl suberimidate (data not shown). Therefore, these bands may represent cross-linked globin, rather than globin attached to membrane components. In addition to these bands and free globin at the dye front, a substantial portion of the

TABLE II

Generation of methemoglobin during treatment with tBHP and H_2O_2 and inhibition by carbon monoxide

Results from a single experiment are given as the spectrophotometrically determined percentage of total hemoglobin in the methemoglobin form in samples treated with oxidant, with and without pre-exposure to CO. The values are averages from calculations using redundant data at several wavelengths, and numbers in parentheses give the S.E.

Oxidant		Percentage of total Hb in metHb form		
tBHP	Concn.:	0	1.0 mM	1.5 mM
−CO		3.3 (1.4)	18.0 (1.4)	44.1 (1.0)
+CO		12.0 (0.1)	18.7 (0.3)	24.7 (0.6)
H_2O_2	Concn.:	0	1.5 mM	3.0 mM
−CO		3.3 (1.4)	54.8 (1.0)	69.3 (1.1)
+CO		12.0 (0.1)	16.7 (0.1)	20.2 (0.04)

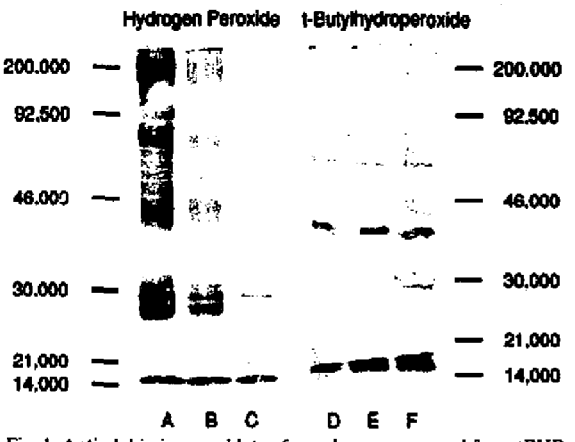

Fig. 1. Anti-globin immunoblots of membranes prepared from tBHP- and H_2O_2-treated red cells. The three lanes on the left are from an experiment using H_2O_2: A, 2.0 mM; B, 1.0 mM; C, untreated control. The three lanes on the right are from an experiment using tBHP: D, untreated control; E, 0.5 mM; F, 1.0 mM. Positions of molecular weight markers are indicated on each side. The bands on the control samples above the low molecular weight region represent residual contaminating antibodies in the rabbit anti-globin serum that react with red cell membrane components.

TABLE III

Effect of tBHP on production of MDA

MDA was measured immediately after a 1 h exposure to oxidant, by analysis of unwashed cell suspensions. Values shown are the mean values above control for n experiments; the S.E. is given in parentheses.

[tBHP]	n	MDA (nmol/g Hb)
0.5 mM	8	2.2 (0.5) *
1.0 mM	8	6.2 (1.5) **
1.5 mM	5	45.1 (15.2) ***

* $0.0005 < P \leq 0.005$, compared to control, untreated cells.
** $0.0005 < P \leq 0.005$ compared to 0.5 mM treatment.
*** $0.01 < P \leq 0.025$ compared to 1.0 mM treatment.

membrane associated globin migrated in the region in which spectrin is found, consistent with previous reports of spectrin-globin cross-linking in H_2O_2-treated cells [22]. Although the extent of anti-globin staining on blots from cells treated with tBHP was less than for H_2O_2-treated cells, for the sample treated at 1 mM tBHP, some staining was detectible in the spectrin region, as well as in a broad band ranging from about 40 to 90 kDa.

In eight experiments, the extent of lipid peroxidation during oxidative treatment with tBHP was monitored by determining the production of TBA-reactive substance, assumed to be MDA, during the 1-h treatment period. (As noted in Methods, interference by other absorbing species prevented accurate determination of MDA production in H_2O_2-treated cells.) As shown in Table III, there was a dose-dependent increase in production of MDA. However, the extent of MDA production relative to control, untreated cells was not very great except at 1.5 mM tBHP, a concentration higher than was used for other studies because it tended to result in hemolysis. Although interference by other chromogens prevented accurate determination of MDA production in H_2O_2-treated cells, the minimal change in absorbance at 532 nm associated with prior washing of the samples suggested that the amount of MDA production in these cells, even those treated with 3 mM H_2O_2, was less than in cells treated with tBHP at 1 mM concentration.

Cell deformability and mechanical stability. The two oxidants also produced distinctly different effects on cellular mechanical properties. As observed by others [22], H_2O_2 treatment caused a profound, dose-related decrease in whole cell deformability, as assayed by osmotic gradient ektacytometry. Cell deformability further decreased after overnight incubation at 37°C, but there was no obvious shift in the position of the curve along the osmolality axis, indicating the absence of major changes in average red cell hydration (Fig. 2a). The deformability loss was primarily due to a reduction in membrane deformability, as shown by measurements using resealed ghost preparations. Increasing concentrations of H_2O_2 caused progressive decrease in the maximum deformation of the ghosts (Fig. 2b). The deformability loss was completely inhibited by pretreatment of the cells with carbon monoxide (CO) to block Hb oxidation (data not shown). It is likely that the marked association of denatured hemoglobin with the membrane of H_2O_2-treated cells may have contributed to their reduced membrane deformability.

In contrast to H_2O_2, treatment with tBHP, followed by overnight incubation, had minimal effect on the deformability of whole red cells or red cell membranes (Fig. 3). The absence of a shift in the osmotic gradient profile also showed that there was no substantive effect on cellular hydration. Although it had minimal or no effect on cell or membrane deformability, in five experiments tBHP caused an increase in the rate of shear induced fragmentation of resealed ghosts prepared

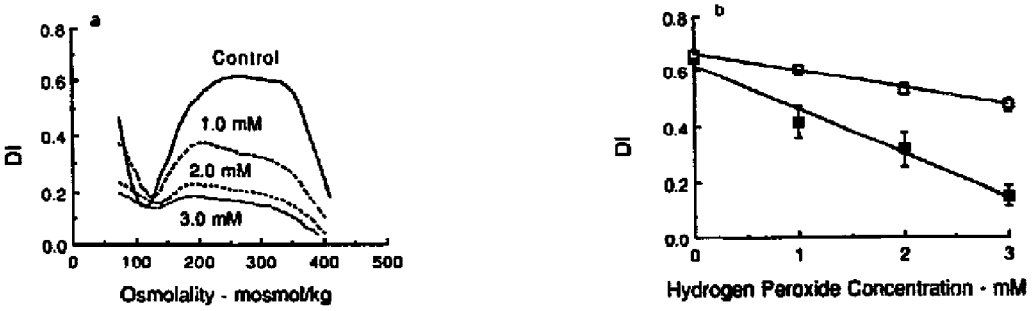

Fig. 2. Effect of H_2O_2 on whole cell and membrane deformability. Cells treated with the indicated concentrations of oxidant and control cells processed in parallel without oxidant were washed once, incubated overnight in BSKG and then assayed using the ektacytometer. (a) Whole cell deformation measured as a continuous function of suspending medium osmolality. The maximum DI decreases markedly with increasing concentration of H_2O_2, but there is no shift of the curve along the osmolality axis, indicating a lack of major alteration in cell water content. (b) Maximum deformation of resealed ghosts as a function of increasing H_2O_2 concentration. □, ghosts prepared and assayed immediately after treatment; ■, ghosts prepared and assayed from cells incubated overnight after treatment. Mean values are plotted for each series of experiments, with error bars representing the S.E. for five experiments without and four with overnight incubation.

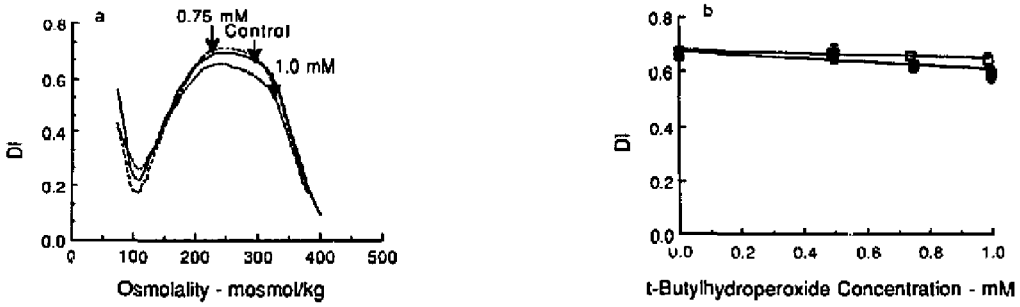

Fig. 3. Effect of tBHP on whole cell and membrane deformability. (a) Whole cell deformability as a function of suspending medium osmolality, performed after overnight incubation. The curves show a slight reduction in maximum deformation in sample treated with 1 mM tBHP, but no shift along the osmotic axis. (b) Maximum deformation of resealed ghosts as a function of increasing tBHP concentration. □, ghosts prepared and assayed immediately after treatment; ■, ghosts prepared and assayed from cells incubated overnight after treatment. Mean values are plotted for each series of experiments, with error bars representing the S.E. for seven experiments without and 3–5 (at the various concentrations) with overnight incubation.

from treated cells. (In one experiment, the fragmentation rate was increased at 0.75 mM tBHP and decreased at 1 mM, but the latter sample had undergone hemolysis during overnight incubation.) In the experiment illustrated in Fig. 4, the time required for the DI signal to fall to half its initial value, a measure of fragmentation rate, was decreased to 2/3 the normal value for membranes from cells treated with 1 mM tBHP, indicating a substantial reduction in the mechanical strength of the membrane. The fragmentation rate for cells treated with H_2O_2 at 1 mM concentration was also more rapid than that for control cells, suggesting that H_2O_2, like tBHP, causes a reduction in membrane yield strength (Fig. 4). However, as the dose of H_2O_2 increased, the apparent rate of fragmentation decreased. This was probably due to reduced membrane deformability at higher H_2O_2 concentrations, since fragmentation rates inherently decrease with decreasing membrane deformation. The obscuring effect of reduced deformability thus prevents quantitative estimates of the change in membrane stability induced by H_2O_2.

Previous work by others had shown that NEM mediated alkylation of sulfhydryl groups on red cell membrane proteins caused an increase in the rate of membrane fragmentation in the ektacytometer [23]. Therefore, we performed experiments to test whether tBHP treatment had reduced the number of free sulfhydryl groups. In three separate experiments, we determined the extent of labeling of spectrin, ankyrin, band 3 and actin by [^{14}C]NEM in tBHP-treated and control cells. When the incorporated radioactivity in each protein band was normalized to the Coomassie blue staining intensity, no significant differences were found in the extent of labeling (data not shown). Moreover, NEM treatment further increased the fragility of tBHP-treated cells (data not shown), further suggesting that the two agents were acting at different sites. We also examined SDS-PAGE gels stained with Coomassie blue for evidence of high molecular weight cross-linked proteins. There was very faint staining of gels from tBHP-treated cells near the top of the gel, indicating very minor cross-linking (data not shown). In general, cross-linking tends to decrease membrane fragility [23],

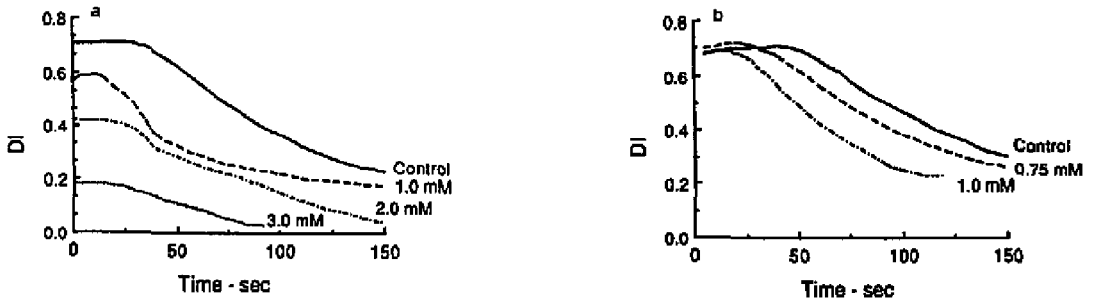

Fig. 4. Effect of H_2O_2 and tBHP on membrane mechanical stability in resealed ghost preparations. Resealed ghosts were prepared after oxidant treatment and overnight incubation and were assayed for their tendency to fragment at high shear stress. (a) Ghosts prepared from H_2O_2-treated cells. (b) Ghosts prepared from tBHP-treated cells. The concentrations of oxidants are indicated on the figure.

TABLE IV

Effect of H_2O_2 and tBHP on binding of autologous IgG to red cells

After treatment, washing and subsequent overnight incubation, paired samples of cells were exposed to either rabbit anti-spectrin or to heat-inactivated autologous serum. Bound IgG was then assayed using ^{125}I-Protein A. Results are expressed as the mean ratio of Protein A counts for treated cells divided by untreated controls. Numbers in parentheses indicate S.E.

Treatment	n	Anti-spectrin	Autologous IgG
H_2O_2			
Control	5	1.0	1.0
1.0 mM	5	1.06 (0.08)	1.09 (0.11)
2.0 mM	6	1.30 (0.24)	1.52 (0.22) *
3.0 mM	4	1.19 (0.15)	1.54 (0.12) **
tBHP			
Control	5	1.0	1.0
0.75 mM	5	1.17 (0.12)	1.12 (0.15)
1.0 mM	4	1.19 (0.11)	1.47 (0.35)

* $0.025 < P \leq 0.05$.
** $0.005 < P \leq 0.025$.

so this is unlikely to be a factor in the increased fragmentation rates of tBHP treated cells.

Effects on binding of autologous serum antibodies. Initial pilot experiments showed that when incubated with autologous serum immediately after oxidant exposure, cells treated with either H_2O_2 or tBHP failed to bind significantly increased quantities of IgG. Because others had reported [24] that some effects of tBHP treatment developed over time after pulse exposure, we subsequently used a procedure in which the cells were washed once after a 1 h exposure to the oxidants and were then incubated overnight at 37°C before exposure to autologous serum. As shown in Table IV, there was still minimal, if any, effect of oxidant treatment on the binding of autologous serum antibody at oxidant concentrations that did not result in hemolysis or increased binding of anti-spectrin. Only cells treated with H_2O_2 at concentrations above 1 mM showed an increase in bound antibody, and this effect was extremely modest.

Discussion

These studies, comparing the effects of H_2O_2 and tBHP under prelytic conditions, reveal distinct differences in the effects of these two agents on important cellular properties. H_2O_2 treatment resulted in increased cation permeability and reduced membrane deformability, whereas tBHP treatment had minimal effect on either property. These differences have not been appreciated in previous work employing more severe treatment conditions, under which some degree of hemolysis occurs. It might be argued that the different effects of the two oxidants reflect only a quantitative difference in the intensity of oxidative damage, rather than a qualitative difference in the targets of damage. Thus, it is important to be certain that tBHP did have access to the cell interior and membrane constituents at the low concentrations at which it was used. Evidence that this was so can be seen from the oxidation of Hb to methemoglobin, the production of MDA and the increase in membrane fragmentation rate that were associated with tBHP treatment. In addition, the increased tendency of the tBHP-treated samples to hemolyze, as compared to H_2O_2-treated samples, indicates significant and a qualitatively different type of membrane damage resulting from exposure to tBHP.

A likely explanation for the large effect of H_2O_2 in reducing cell deformability is the extensive association with and cross-linking of hemoglobin to the membrane, as already proposed by others [22]. Whether this general process contributes to the membrane permeability defect is not known.

A mechanism to explain the effect of tBHP in enhancing membrane fragility is less easy to formulate. We tested the possibility that it might be due to alkylation of membrane sulfhydryl groups, since others had shown that alkylation of sulfhydryl groups by NEM has no effect on membrane deformability but enhances fragmentation rates [23]. However, in accord with recent findings by investigators using a different assay [25], we found no evidence for reduced availability of membrane sulfhydryl groups in tBHP-treated cells. Trainor et al. [26] have reported that H_2O_2-induced cross-linking of globin to spectrin impairs the normal tetrameric association of spectrin, which might be expected to result in reduced membrane stability. Anti-globin immunoblotting suggested that treatment with 1 mM tBHP induced a small amount of globin binding to spectrin. It may be that a small amount of globin-spectrin association could have resulted in decreased membrane stability in tBHP-treated cells, whereas the larger amount in H_2O_2-treated cells caused impaired membrane deformability as well. Another possible mechanism could involve interaction of the membrane with hemin, which can be released when hemoglobin undergoes oxidative denaturation [3]. It has been shown that micromolar concentrations of hemin can induce increases in membrane fragility similar to those found in the current study [3,27].

Our findings that tBHP had no effect on membrane permeability or membrane deformability are at variance with two previous reports from other laboratories [24,28]. However, Ney et al. also found minimal effect of tBHP on K efflux rates when cells were treated at concentrations below 1 mM [25]. The important variable in these different studies is the ratio of tBHP to red cells. Since tBHP is a lipophilic agent, the amount of oxidant relative to the quantity of membrane pre-

sent is especially important in determining the level of exposure of membrane components to the oxidant. The experiments in which permeability and deformability changes were observed used lower hct and/or higher concentrations of tBHP and thus represented substantially higher treatment levels. This is further indicated by the presence of substantially more membrane protein cross-linking in the study by Corry et al. [28], as compared to the current results and those of Ney et al. [25]. It is of interest that despite this lack of effect on cation permeability, tBHP treatment was associated with an increased tendency to hemolyze, as compared to H_2O_2 treatment. The mechanism of hemolysis in tBHP-treated cells is not known, but it appears not to be simply due to an increase in membrane permeability.

In contrast to their effects on other membrane properties, neither of the two types of oxidative treatment produced a substantial increase in the ability of the cells to bind autologous serum antibodies. A small increment in antibody binding appeared at higher levels of H_2O_2 treatment, but this represented only a 50% increase at an oxidant concentration that could be tolerated without hemolysis or exposure of intracellular antigens. This result contrasts with those reported by Beppu et al. who found that oxidation in the presence of iron catalysts resulted in extensive binding of autologous serum anti-Band 3 [29]. It may be that the specific oxidative pathways involved are critical in determining whether oxidative damage causes increased binding of autologous serum IgG. Several features suggest that the oxidative damage inflicted by the iron catalysts proceeds by yet a different pathway than those initiated by tBHP or H_2O_2.

These observations illustrate the need for detailed understanding of cellular effects of oxidative treatment in trying to assess the role of oxidative damage in physiological processes, including the generation of sites for binding of autologous serum antibodies. Further definition of the oxidative mechanisms involved and their relationship to the types of cell damage that result should provide a basis for better understanding the role of different types of oxidant damage in normal and pathologic red cell changes.

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