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Progressive oxidative membrane damage in erythrocytes after pulse treatment with *t*-butylhydroperoxide *

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Development of membrane damage in erythrocytes in the presence of the radical-forming oxidant *t*-butylhydroperoxide is a well established fact (see, for example, Deuticke et al. (1986) Biochim. Biophys. Acta 854, 169–183). We have now demonstrated that a mere pulse treatment of erythrocytes (5–15 min) with this agent leads to subsequent development of progressive oxidative membrane damage in spite of the absence of exogenous oxidant. Damage comprises the occurrence of ion leakiness and subsequent colloid-osmotic lysis, enhancement of the transbilayer mobility of phospholipid analogues, and lipid peroxidation. There is, however, only very little concomitant oxidation and precipitation of hemoglobin. Defect formation is not due to oxidation of SH-groups nor is it directly related to lipid peroxidation, since it can be suppressed by thiourea without concommitant inhibition of lipid peroxidation. This 'spontaneous' development of membrane damage can be antagonized by metabolic substrates and by desferrioxamine, indicating that lack of protective metabolic resources as well as the presence of catalytic metal (iron) complexes are required for the development of membrane damage. This progressive development of injury in cells only temporarily exposed to an exogenous oxidant may be regarded as a more appropriate model for oxidative membrane damage under pathophysiological conditions in vivo than cells exposed to continuous damage by exogenous oxidants.

Introduction

The damage inflicted on mammalian cell membranes by oxygen-derived radicals and radical-producing treatments has been studied intensively during the past years [1–4]. A favorite object of such studies has been the red blood cell which contains systems catalyzing the formation and the degradation of oxy-radicals [5,6]. Oxidative membrane damage has been ascribed to modification of proteins [7–9] or peroxidation of lipids [5,10]. The relative contribution of the two processes is a matter of debate [11] and may well depend on the system chosen as well as on the ambient conditions. In a recent study [12] we have characterized to some detail aqueous membrane leaks formed in erythrocytes treated with a lipoperoxide analogue, *t*-butylhydroperoxide, but could not clarify the

* Dedicated to Professor E. Gerlach, Munich, on the occasion of his 60th anniversary.

Abbreviations: DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonate; DNDS, 4,4′-dinitrostilbene-2,2′-disulfonate.

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final mechanism of leak formation. Van der Zee et al. [9] have recently reported data indicating that membrane leak formation by *t*-butylhydroperoxide may result from oxidative damage to proteins rather than from lipid peroxidation.

t-Butylhydroperoxide is activated in erythrocytes by reacting with hemoglobin and other heme iron complexes, giving rise to radicals [13–18] which induce the membrane alterations outlined above. In principle, such oxidative membrane changes are of interest as a model for red cell membrane changes in hematological diseases. On the other hand, the progressive and extensive oxidation of hemoglobin in *t*-butylhydroperoxide-treated cells [13–15], which is an obligatory consequence of the formation of *t*-butyloxy radicals, has no counterpart (to this extent) in erythrocytes undergoing oxidative damage in pathological states. It would therefore be desirable to find an experimental setup under which oxidative membrane damage occurs while oxidation of hemoglobin is avoided. In the following it is shown that the formation of aqueous leaks (and flip sites for phospholipid analogues) as well as lipid peroxidation, but not hemoglobin oxidation, continue progressively in cells only pretreated briefly with *t*-butylhydroperoxide and than incubated in absence of the radical forming agent. The characteristics of the membrane damage initiated by pretreatment with *t*-butylhydroperoxide and its sensitivity to antioxidants will be described.

Materials and Methods

Materials

Human blood from healthy donors was from the local blood bank. *t*-Butylhydroperoxide was from Fluka, Neu-Ulm. Butylated hydroxytoluene, diphenylamine, 4,4′-dithiodipyridine, bathophenanthroline disulfonate, diethyldithiocarbamate (DDC), and 4,4′-diisothiocyanostilbene-2,2′-disulfonate were from Sigma, Munich; 4,4′-dinitrostilbene-2,2′-disulfonate (DNDS) form K & K Chemicals, Desferal (desferrioxamine methanesulfonate) from CIBA, Basel, Dextran 4 from Serva, Heidelberg and Dextran 1 (Promit®) from Schiwa, Glandorf, F.R.G. ^{36}Cl was from Amersham-Buchler, Braunschweig, and [1-^{14}C] palmitoylcarnitine chloride from New England Nuclear, Dreieich.

Methods

Freshly taken human blood, anticoagulated with citrate, was stored at 4° C and used for experiments within 5 days. Erythrocytes were isolated by centrifugation (5 min, 6000 × *g*), plasma and buffy coat removed and the cells washed three times with isotonic saline at room temperature.

Oxidative treatment

Washed erythrocytes were suspended in 10 volumes of the following medium (concentrations in mM): KCl (90), NaCl (40), NaH_2PO_4/Na_2HPO_4 (12.5), trisodium citrate (20), sodium azide (5.0) (= Medium B). Impermeable citrate served to protect the cells against colloid-osmotic lysis upon formation of leaks permeable to small ions, azide suppresses precipitation of non-intact [14,15] hemoglobin [12].

Exposure to *t*-butylhydroperoxide was started by adding another 10 volumes of medium A (= medium B without azide) containing the oxidant at twice the desired final concentration. The suspensions were incubated at 37° C, usually for 15 min, in a shaking water bath. After this pretreatment the suspension was rapidly cooled to 0–2° C and centrifuged. The cells were washed three times at 0–2° C in 5 vols of medium A, resuspended in 12 vols of medium A (pH 7.4) containing 2.5 mM sodium azide (or potassium cyanide) unless indicated otherwise, adjusted to 37° C and incubated for the time periods desired. Further additives are indicated at the respective results. At the end of this 'main incubation period' butylated hydroxytoluene, dissolved in a small volume of ethanol, was added at a final concentration of 100 μM to stop lipid peroxidation and leak formation [12]. A control sample was already stopped at the start of the main incubation period to obtain the initial levels of membrane alterations. Either the total suspensions, or the cells washed twice in medium A, were immediately used for further analyses.

Characterisation of the modified cells

(a) Oxidative changes of hemoglobin. At appropriate intervals 50 μl of the cell suspension

incubated as described above were diluted into 3 ml of 75 μM phosphate buffer (pH 7.4) containing 2.5 mM NaN_3. From the absorbances at 560, 577, 630, and 700 nm the fractional amounts of oxidized hemoglobin were calculated using the formulae given by Szebeni et al. [19], taking into account the different absorption coefficients of azidomethemoglobin as compared to methemoglobin [20].

(b) Formation of malondialdehyde. Malondialdehyde, taken to represent the thiobarbituric-acid-reactive material, was determined in the whole cell suspensions at 37°C by the procedure of Stocks and Dormandy [21]. The absorbance A_{532} was converted into amounts of malondialdehyde formed per ml of cells using the hematocrit value and a calibration curve obtained by reacting malondialdehyde, freshly prepared from 1,1',3,3'-tetraethoxypropane and HCl, with the reagents required.

(c) Disappearance of phospholipids. Cells treated as described above were lysed by 2 vols. of H_2O. 2 vols. of lysate were extracted with isopropanol/chloroform [22]. After filtration the extracts were evaporated to dryness, taken up in 100–200 μl chloroform and spotted on precoated silica 60 F plates (20 × 20 cm, Merck, Darmstadt, No. 5715). Phospholipids were separated by two-dimensional TLC using chloroform/methanol/25% NH_3/water (90 : 54 : 6 : 5, v/v) in the first dimension and chloroform/methanol/acetic acid/water (60 : 30 : 12 : 2, v/v) in the second dimension. Spots localized by molybdate spray were scratched from the plates and phospholipid fractions quantified by phosphorus determination [23].

(d) Changes of membrane SH-groups and binding of heme compounds to the erythrocyte membrane. Washed cells were lysed in 40 vols. of 5 mM phosphate buffer (pH 8) at 4°C. Membranes, washed three times in the buffer, were packed by centrifugation (30 000 × *g*, 10 min) and solubilized by addition of 6 vols of the buffer containing 1% (w/v) SDS. 1 ml of the solubilized membranes was mixed with 50 μl of a 3 mM aqueous solution of 4,4'-dithiodipyridine. After warming to 37°C for 15 min and a subsequent 15 min incubation at room temperature the absorbances were read at 324 nm and 406 nm against appropriate blanks. The protein content in the samples was determined [24], using bovine serum albumin (Sigma, protein standard P6029) as a reference. The SH-content of the samples was calculated from A_{324}, using glutathione solutions for calibration. A calibration curve for bound hemoglobin was obtained from standard solutions of hemoglobin, containing SDS at the concentration also used for solubilization. This was essential, since SDS at this molar ratio converts all of the hemoglobin into hemichrome. The concentrations of bound hemoglobin, calculated from A_{406}, were expressed as (bound heme protein) per (total protein minus bound heme protein).

(e) Membrane protein patterns. Ghost membranes prepared as described above were solubilized in buffer (0.3 M Tris (pH 7)/5% (w/v) SDS) [25] in the presence of 5 mM *N*-ethylmaleimide [26]. After addition of 20% (v/v) glycerol, samples containing between 0.2 and 0.8 mg protein/ml were incubated at 37°C for 50 min. When desired, disulfide bonds were split by incubating the ghosts with 40 mM dithioerythritol for 30 min at 37°C. Subsequently, 50 mM *N*-ethylmaleimide were added to prevent aggregation of protein material [26]. 30 μg of membrane protein were applied per slot of a gradient slab gel. The stacking and the running gel, the Tris-glycine buffer and the running conditions were precisely as described by Lugtenberg et al. [27]. The running gel was a linear 5 to 20% acrylamide gradient gel containing acrylamide and bismethyleneacrylamide in the proportion given by Lugtenberg et al. [27]. The gels were stained overnight with 0.25% Coomassie brilliant blue R 250 in 50% ethanol/10% acetic acid. Destaining was with 30% methanol/10% acetic acid.

(f) Leak permeability: Tracer fluxes. Increases of membrane leak permeability were routinely quantified by measuring the increase of the stilbenedisulfonate-insensitive Cl^--permeability at 0°C using tracer fluxes as described earlier [12]. At 0°C this permeability is extremely low ($P < 10^{-9}$ cm · s^{-1}) in control cells.

(g) Leak permeability: Lysis studies. Leak formation was also characterized by following the colloid-osmotic lysis of damaged cells upon suspension in isotonic saline (KCl 100 mM, NaCl 50 mM) as described previously [28]. The apparent size of the leaks was estimated by testing nonelec-

trolytes of varying molecular size for their ability to act as osmotic protectants [12]. Cells were suspended in isotonic saline containing 40 mosmol/l of either mannitol, sucrose, raffinose, Dextran 1 ($M_r = 970$) or Dextran 4 ($M_r = 4000$–6000) and incubated for 20 h at 4° C. The extent of lysis was then determined. The size of the induced holes was set equal to the radius of the nonelectrolyte protecting 95% of the cells against lysis for 20 h. Radii of protecting solutes were adopted from Ref. 29.

(h) Transbilayer reorientation of palmitoylcarnitine. The transbilayer reorientation (flip rate) of exogenous palmitoylcarnitine was measured by a procedure described earlier [30] for lysophospholipids. Briefly, [^{14}C]palmitoylcarnitine was inserted into the outer membrane layer of erythrocytes and its slow transbilayer reorientation to the inner membrane layer quantified by following the loss of extractability of the label from the cells by fatty acid-free bovine serum albumin at 37° C. Bovine serum albumin removes single chain amphipaths selectively from the outer layer of the erythrocyte membrane.

Results

(A) Development of membrane damage in erythrocytes after pretreatment with t-butylhydroperoxide

Human erythrocytes exposed briefly (between 5 and 15 min) to *t*-butylhydroperoxide in the presence of azide at 37° C undergo only little peroxidation and formation of membrane leaks, while about 15–30% of the hemoglobin are oxidized [12]. If these pretreated cells are washed thoroughly in the cold and then incubated in fresh medium without added oxidant ('main incubation period'), the stilbenedisulfonate-insensitive permeability to chloride – as a measure of membrane leak permeability – increases progressively at a considerable rate (Fig. 1A). Azide was added to the media not only for the pretreatment, but also during the main incubation period. Its requirement during the pretreatment has been explained above. During the subsequent 'main incubation period' it is not a necessary additive but markedly enhances the extent of damage. Addition of cyanide has the same effect (Fig. 1A).

In parallel to leak formation, phospholipids are peroxidized progressively as indicated by the formation of malondialdehyde (Fig. 1B) and degradation of aminophospholipids (Fig. 2) (cf. Refs. 10 and 16). On the other hand, there is only very

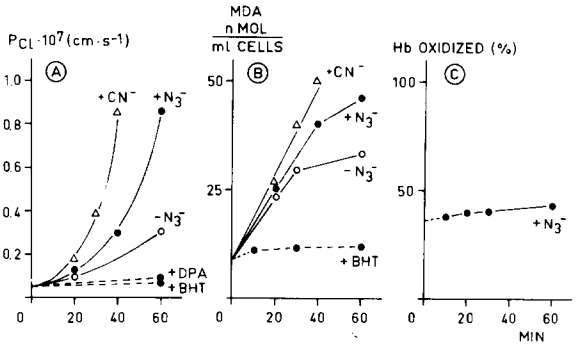

Fig. 1. Time-dependent development of oxidative damage in human erythrocytes pretreated with *t*-butylhydroperoxide. Cells were first exposed to *t*-butylhydroperoxide (2 mM) in the presence of N_3^- (2.5 mM) for 15 min, washed at 0° C and resuspended in Medium A (37° C, pH 7.4) as described in Materials and Methods. '0 min' corresponds to the moment of resuspension. Additives: N_3^- or CN^- 2.5 mM, BHT (butylated hydroxytoluene) 100 μM, DPA (diphenylamine) 100 μM. Incubations were terminated by addition of butylated hydroxytoluene and the various parameters determined as described in Materials and Methods. (A) Leak permeability (P_{Cl}) defined as stilbenedisulfonate-insensitive chloride permeability at 0° C. (B) Lipid peroxidation quantified by formation of malondialdehyde (MDA). (C) Oxidation of hemoglobin to methemoglobin and non-intact [14,15] hemoglobin.

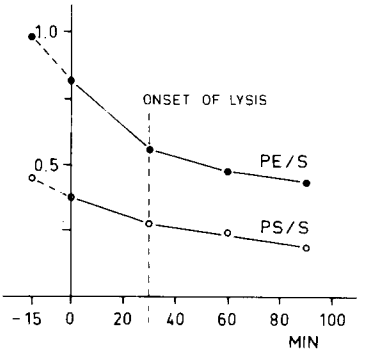

Fig. 2. Changes in the content of phosphatidylethanolamine (PE) and phosphatidylserine (PS) in erythrocytes pretreated with *t*-butylhydroperoxide, during subsequent incubation at 37° C in the presence of 2.5 mM N_3^-. Incubation terminated after different time periods by addition of 100 μM butylated hydroxytoluene. '−15 min' refers to the start of the sensitization period. Content of the aminophospholipids normalized to that of sphingomyelin (S), which is resistant to peroxidative degradation.

little progress in the oxidation of hemoglobin during the main incubation period (Fig. 1C), in contrast to the situation in the presence of *t*-butylhydroperoxide [12]. The oxidized forms of hemoglobin (methemoglobin and non-intact [14,15] hemoglobin) increase only by a few percent. The extent of leak formation, lipid peroxidation (and of oxidation of hemoglobin) increases with increasing duration of the pretreatment and is related to the extent of the primary oxidative stress in the presence of *t*-butylhydroperoxide (Table I). Similar effects can also be initiated by a brief pretreatment of cells with H_2O_2 in the presence of azide and with *t*-butylhydroperoxide at lower temperatures, e.g. 15 °C, in the absence of azide (data not shown). Time-course and extent of leak formation and lipid peroxidation vary considerably from donor to donor, but are reproducible in the same batch of stored blood.

Hemoglobin, although only oxidized to a minor extent during the pretreatment (cf. Fig. 1C and Table I), attaches progressively to the membrane during the subsequent incubation period. Hemoglobin precipitation is somewhat less pronounced, however, during the secondary peroxidation than during a primary treatment with *t*-butylhydroperoxide (Fig. 3). Azide suppresses this hemoglobin attachment.

Peroxidative processes are involved in the progress of membrane damage after removal of *t*-butylhydroperoxide since antioxidants such as butylated hydroxytoluene or diphenylamine almost completely abolish the increase of leak formation and of lipid peroxidation during the main incubation period (Fig. 1).

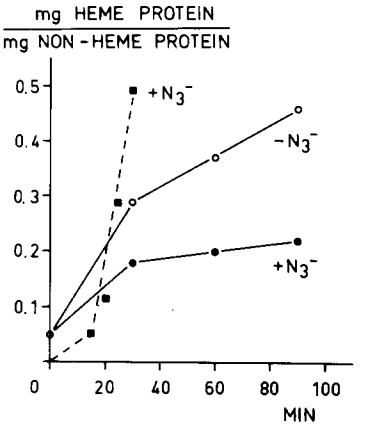

Fig. 3. Time-dependent attachment of heme protein to the membrane in cells pretreated with *t*-butylhydroperoxide during subsequent incubation at 37 °C (solid lines). Heme attachment during incubation in the presence of *t*-butylhydroperoxide (dashed line) is shown for comparison.

Oxidation of membrane SH-groups is probably not involved in leak formation in pretreated cells although there is a 40% decrease of the content of SH-groups during 90 min main incubation (from 110 to 66 nmol SH/mg membrane protein). The analyses were complicated by the precipitation of hemoglobin at the membranes. In our calculations it was assumed that the attached pigment does not contain free thiols since it consists essentially of

TABLE I

INFLUENCE OF THE DURATION OF A PRETREATMENT OF ERYTHROCYTES WITH *t*-BUTYLHYDROPEROXIDE (2 mM) AND N_3^- (2.5 mM) ON THE DEVELOPMENT OF OXIDATIVE DAMAGE DURING A SUBSEQUENT INCUBATION IN THE ABSENCE OF *t*-BUTYLHYDROPEROXIDE

Cells were pretreated at 37 °C, pH 7.4 for the time indicated, washed three times at 0 °C with medium A and then incubated in medium A (pH 7.4, 37 °C). Incubation terminated by addition of 100 μM butylated hydroxytoluene and cooling of the sample to 0 °C. (Data from one experiment out of 3 with the same result).

Conditions of incubation	P_{Cl} (10^{-7} cm·s^{-1})			Malondialdehyde (nmol/ml cells)			Hemoglobin oxidized (%)		
Duration of pretreatment:	5 min	10 min	15 min	5 min	10 min	15 min	5 min	10 min	15 min
0 min	0.05	0.06	0.06	1	5	7	4	17	30
60 min	0.05	0.20	0.37	25	46	45	7	21	37
60 min with N_3^- (2.5 mM)	0.16	0.75	1.15	56	67	68	15	28	41

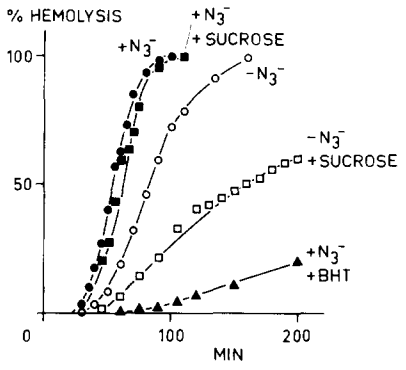

Fig. 4. Progress of colloid-osmotic lysis due to formation of membrane leaks in erythrocytes after treatment with *t*-butylhydroperoxide (2 mM) and N_3^- (2.5 mM). After washing, the cells were incubated at 37°C in KCl (100 mM), NaCl (50 mM), containing as additives: N_3^- (2.5 mM), BHT (butylated hydroxytoluene (100 μM)), or sucrose (30 mM) in various combinations. The curves obtained in the presence of butylated hydroxytoluene reflect the extent of leakiness achieved during the pretreatment with *t*-butylhydroperoxide.

irreversible hemichromes [31]. The decrease in SH-content can be fully reversed by reductive treatment with dithioerythritol at least up to a 30 min main incubation period. The normalization of the SH-groups, however, is not accompanied by a restoration of the normal permeability (data not shown).

Cells pretreated with *t*-butylhydroperoxide undergo progressive lysis during subsequent incubation in the absence of the oxidant (Fig. 4). This indicates the formation of rather unselective, presumably aqueous, membrane leaks. The rate of lysis can be enhanced by azide (and cyanide) and is diminished, although not fully abolished, by butylated hydroxytoluene. The sensitivity to these modifiers can only be explained by changes in rates of leak formation but not by changes of fluxes via existing leaks. On the other hand, rates of lysis can be diminished by sucrose in cells incubated in the absence of azide. Lysis is therefore colloid-osmotic, i.e., the loss of the normal, very low, cation permeability leads to an uptake of salt and water, driven by the osmotic drag of intracellular impermeant solutes (hemoglobin, etc.) (for references, see Ref. 34). This type of lysis can be prevented if the drag is counterbalanced by extracellular impermeant solute. Partial protection against lysis by sucrose, in the absence but not in the presence of azide indicates that in the former case a single leak has about the diameter of sucrose while in the latter case the leak is much larger than sucrose. On the basis of this principle more detailed information on the leak size was obtained. Cells were pretreated with *t*-butylhydroperoxide for 15 min, washed and incubated further in the absence of the peroxide for different time intervals (see Fig. 5). Leak formation was then stopped by butylated hydroxytoluene and the apparent leak size determined according to the principle outline above (see also Ref. 34) as described in Materials and Methods. As shown in Fig. 5, the leak size seems to increase with the length of the main incubation period. While after 30 min Dextran 1 ($M_r = 1000$, spherical radius = 0.85–1.0 nm) still provides 95% protection, Dextran 4 or even larger compounds are required after 45 and 60 min main incubation time. This indicates apparent radii of at least 1 nm after 30 min and of more than 1.5 nm after 60 min.

Treatment of cells with *t*-butylhydroperoxide induces alterations of membrane proteins indicated by formation of high molecular weight aggregates in SDS-gel electrophoresis [25,32]. Minor

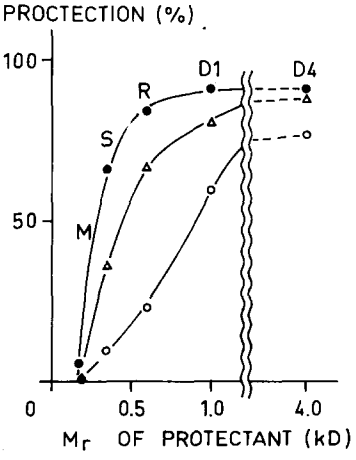

Fig. 5. Estimation of the size of the leaks formed in *t*-butylhydroperoxide-pretreated cells during subsequent incubation at 37°C in peroxide-free media for different intervals: ○, 30 min; △, 45 min; ●, 60 min. After this incubation the progress of damage was terminated by butylated hydroxytoluene (100 μM). Cells were then incubated in KCl/NaCl (100/50 mM) in the presence of the various protectants (40 mM) for 20 h as described in Materials and Methods. M mannitol; S, sucrose; R, raffinose; D1, Dextran 1000; D4, Dextran 4000.

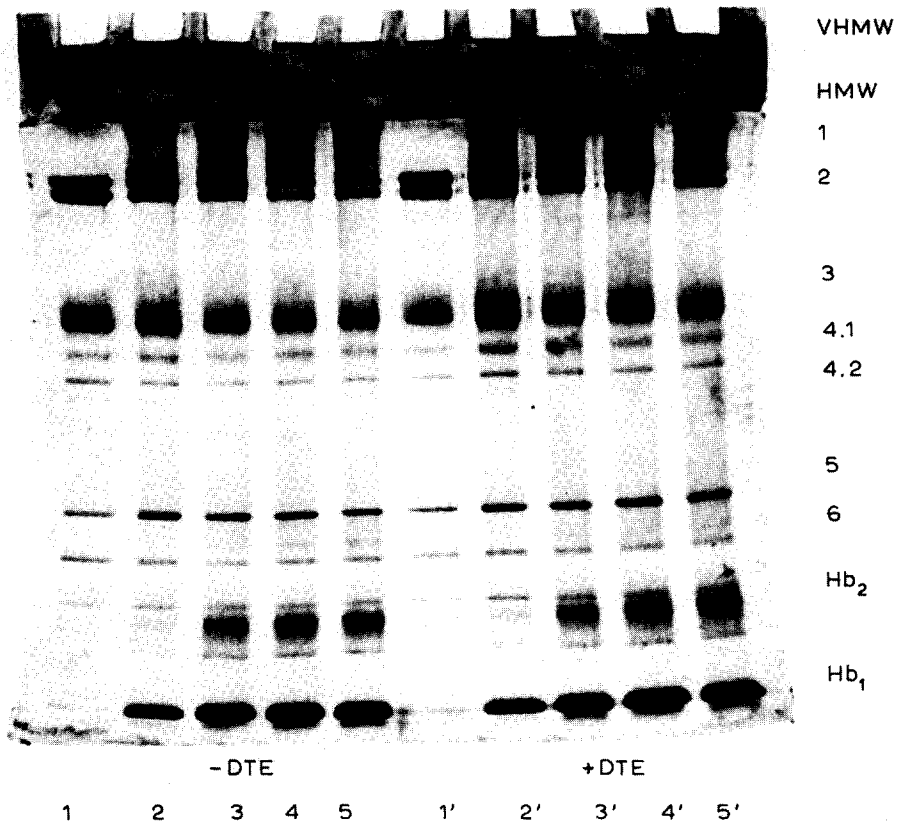

Fig. 6. Membrane polypeptide patterns of erythrocytes incubated after pretreatment with *t*-butylhydroperoxide. Human erythrocytes (Slot 1,1′) were exposed to *t*-butylhydroperoxide as described in Methods, washed and incubated further (with added azide) at 37°C for 0 min (2,2′), 30 min (3,3′), 45 min (4,4′) and 60 min (5,5′). After termination of the reaction by butylated hydroxytoluene, cells were washed and lysed, ghosts were isolated and membranes solubilized and treated with *N*-ethylmaleimide as described in Materials and Methods. 30 μg of membrane protein (corrected for bound hemoglobin) were applied per slot. Slot 1′–5′: the solubilized membrane proteins were reduced with 40 mM dithioerythritol followed by addition of 50 mM *N*-ethylmaleimide [26]: HMW, high molecular weight material; VHMW, very high molecular weight material. Nomenclature of peptide bands according to Ref. 59.

amounts of high molecular weight and very high molecular weight material not even penetrating into the stacking gel are present already after the short (15 min) period of pretreatment. Their amounts increase only slightly during the subsequent main incubation (Fig. 6) in the absence of *t*-butylhydroperoxide. These high molecular weight materials do not disappear upon treatment of the solubilized ghosts with dithioerythritol. They seem to be formed at the expense of spectrin (band 1 + 2) and, possibly, of band 3. Increasing precipitation of hemoglobin on the membrane during the main treatment is also evident from the gels.

Membrane damage occurring in the presence of *t*-butylhydroperoxide as well as in pretreated cells enhances the transbilayer mobility of single-chain amphipathic compounds. Palmitoylcarnitine recently proved very useful as a probe (J. Classen (1986), MD Thesis, RWTH Aachen). The rates of flip-flop of this compound increase progressively (Fig. 7) in cells treated or only pretreated with *t*-butylhydroperoxide, supporting the hypothesis [30,33,34] that the induced leaks may also act as transbilayer reorientation sites for phospholipids.

(B) Modifiers of the damage developing in t-butylhydroperoxide-pretreated cells

(1) Chelators. The damage developing in *t*-

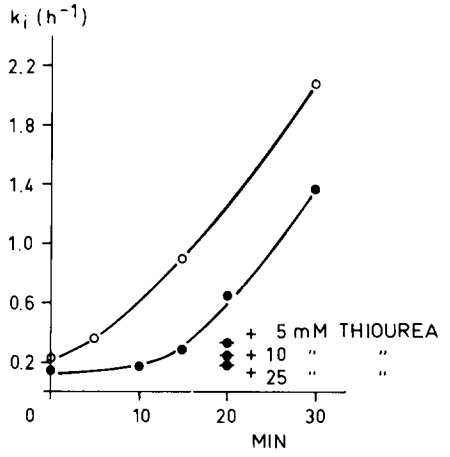

Fig. 7. Enhancement of the transbilayer reorientation of palmitoylcarnitine in erythrocytes treated with 2 mM *t*-butylhydroperoxide and 2.5 mM azide at 37 °C for the time periods indicated on the abscissa (●) or pretreated for 15 min with these agents, washed and than incubated without oxidant (○). Oxidative reactions were interrupted by addition of 100 μM butylated hydroxytoluene and flip rates measured as described [30]. ●, Treated with *t*-butylhydroperoxide in the presence of thiourea; k_i, initial rate of reorientation.

TABLE II

PROTECTIVE INFLUENCE OF CHELATING AGENTS AND GLYCOLYTIC SUBSTRATES ON THE DEVELOPMENT OF OXIDATIVE DAMAGE IN ERYTHROCYTES PRETREATED WITH *t*-BUTYLHYDROPEROXIDE

Cells were exposed to the oxidant and azide (2.5 mM) for 15 min, washed, resuspended in medium A and incubated at 37 °C for 20 and 40 min. DIDS-insensitive fluxes of Cl^- (ΔP_{Cl}) and levels of malondialdehyde (ΔMDA) were measured as described in materials and Methods. Values refer to the increase of both parameters between the two time points and are normalized to the controls without any additive. Mean values ± S.E., number of experiments in parentheses. n.d., not determined.

Additive	$\frac{\Delta P_{Cl}}{\Delta P_{Cl,control}}$	$\frac{\Delta MDA}{\Delta MDA_{control}}$
None (control)	1.0	1.0
Desferrioxamine 2 mM	0.16 ± 0.06 (9)	0.15 ± 0.04 (8)
Diethyldithiocarbamate 2 mM	0.05 ± 0.04 (4)	0.01 ± 0.02 (4)
Iodoacetate 2 mM	1.00 ± 0.07 (7)	1.32 ± 0.21 (7)
Glucose 5 mM	0.35 ± 0.10 (3)	0.64 ± 0.13 (4)
Glucose + iodoacetate	1.25 ± 0.27 (3)	1.48 (2)
Inosine 5 mM	0.39 ± 0.21 (5)	0.54 ± 0.14 (4)
Inosine + iodoacetate	1.42 ± 0.48 (3)	1.82 ± 0.90
2-Deoxyglucose 5 mM	0.75 ± 0.12 (5)	n.d.
Deoxyglucose + iodoacetate	1.01 ± 0.25 (5)	n.d.

butylhydroperoxide-pretreated cells is likely to result from a free-radical chain reaction primarily initiated by the butyloxy radicals. Various forms of iron are presumed to act as catalysts in such propagation processes (for references, see Refs. 2 and 3). For the present case this follows from the protective action of desferrioxamine (Table II), a rather specific chelator of ferric iron [35]. Two other chelators, EDTA and bathophenanthroline disulfonate, were not inhibitory (data not shown). Leak formation and lipid peroxidation were also suppressed by diethyldithiocarbamate, known to act as an antioxidant [36] and an inhibitor of superoxide dismutase [4].

(2) Metabolic protection. Cells pretreated with *t*-butylhydroperoxide can protect themselves to some extent against the damage developing during subsequent incubation without the oxidant by metabolic processes sustained by glucose and inosine (Table II). Protection is abolished by iodoacetate, which excludes that inosine itself might act as a scavenger [37]. 2-Deoxyglucose has only a minor protective influence. This indicates that reducing equivalents produced from deoxyglucose metabolism [38] are insufficient to suppress the events leading to oxidative damage. Metabolic substrates also prevent the slow oxidation of hemoglobin during the main incubation period (data not shown).

(3) Protection by thiourea. Thiourea is known as a hydroxyl radical scavenger [39,40]. It can also serve, however, as an antioxidant since it is readily oxidized by alkoxy radicals [40] and by agents such as H_2O_2 [41] or periodate (Heller, K.B., unpublished results). Thiourea has recently been reported to suppress leak formation by *t*-butylhydroperoxide but not by H_2O_2 [9]. We have analyzed this effect in some detail (Figs. 8A, C). Thiourea protects cells exposed to *t*-butylhydroperoxide in a saturating fashion. 50% suppression of leak formation require less than 1 mM. Besides leak formation, the enhancement of transbilayer reorientation of palmitoylcarnitine is also suppressed by thiourea (cf. Fig. 7).

Concerning the effects of thiourea on lipid peroxidation by *t*-butylhydroperoxide previous re-

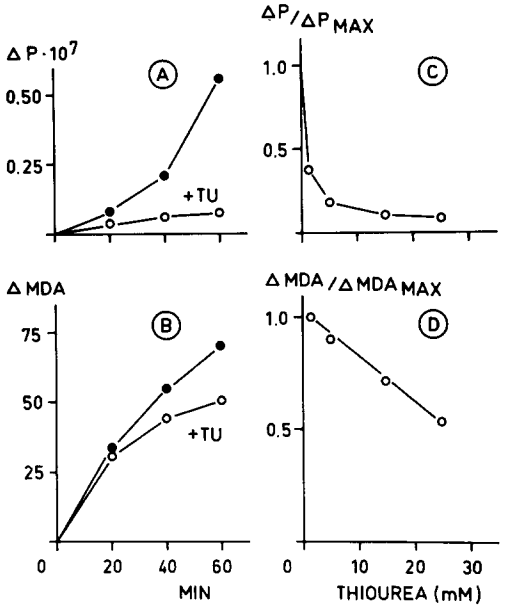

Fig. 8. Influence of thiourea on the oxidative damage of erythrocytes during treatment with *t*-butylhydroperoxide (2 mM) and N_3^- (2.5 mM). (A, B) Time-course of the increase of the stilbenedisulfonate-insensitive Cl-permeability (ΔP (10^{-7} cm·s^{-1})) and of malondialdehyde (ΔMDA (nmol·ml cells^{-1})) in the absence (●) or in the presence (○) of 15 mM thiourea. (C, D) Dose response curves. Cells treated with oxidant and thiourea for 40 min at 37 °C. Incubation terminated by 100 μM butylated hydroxytoluene. Malondialdehyde values corrected for interference of thiourea with the thiobarbituric acid reaction. Mean values from 2–4 experiments normalized to values in the absence of thiourea. MDA, malondialdehyde.

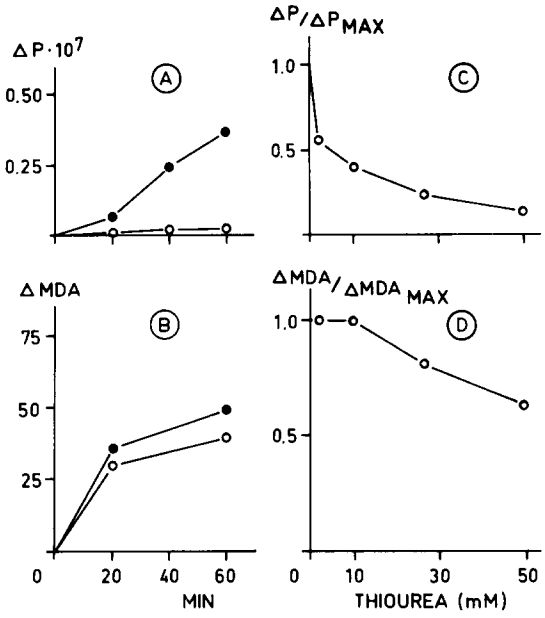

Fig. 9. Influence of thiourea on the oxidative damage developing in erythrocytes pretreated with *t*-butylhydroperoxide (2 mM) and N_3^- (2.5 mM), during a subsequent incubation at 37 °C. (A, B) Time-course in the absence (●) or presence (○) of 50 mM thiourea. (C, D) Dose response curve for a 60 min incubation. Mean values from 2–4 experiments normalized to values in the absence of thiourea. MDA, malondialdehyde.

ports are contradictory. Marked inhibition [15,25] as well as a lack of influence [9] have been reported. Parts of this contradiction may result from the fact that thiourea interferes with the thiobarbituric acid assay. Increasing concentrations of thiourea suppress colour development to an increasing extent (Heller, K.B., unpublished data). When this artifact is corrected for, formation of malondialdehyde in the presence of *t*-butylhydroperoxide turns out to be affected very little by thiourea at concentrations up to 5 mM (Figs. 8B, D), although at higher levels lipid peroxidation becomes suppressed.

In cells incubated after a mere pretreatment with *t*-butylhydroperoxide thiourea also inhibits leak formation while having little effect on lipid peroxidation up to 10 mM (Figs. 9A–D). Only at higher concentrations formation of malondialdehyde is also inhibited. The disappearance of phosphatidylethanolamine is also less pronounced in the presence of 50 mM thiourea (data not shown). On the other hand, SH-group oxidation during incubation of pretreated cells is not affected by thiourea, nor are the electrophoretic polypeptide patterns any different from those in the absence of thiourea. Finally, neither the slow oxidation of hemoglobin nor the progress of its precipitation on the membrane are altered by thiourea (data not shown).

Discussion

The data presented here indicate that a brief exposure of erythrocytes to *t*-butylhydroperoxide initiates a peroxidative chain reaction in the erythrocyte membrane which proceeds after the initiating reagent has been removed, while the progress of oxidative degeneration of hemoglobin is only minimal (Fig. 1). Virtually complete removal of *t*-butylhydroperoxide after the pretreat-

ment may be assumed since the cells were washed thoroughly so that a highly permeable and water-soluble compound like *t*-butylhydroperoxide should have been removed completely. Increasing the number of washing cycles and addition of albumin which should bind *t*-butylhydroperoxide did not diminish the secondary peroxidation and leak formation.

Furthermore, traces of *t*-butylhydroperoxide would not be sufficient to produce the changes observed. Below 0.5 mM *t*-butylhydroperoxide does neither induce lipid peroxidation nor leak formation in suspensions containing red cells at a hematocrit of 5% [12].

Finally, in the presence of *t*-butylhydroperoxide much more hemoglobin is oxidized in parallel to a given increase of lipid peroxidation or leak formation than in the secondary damage described here. According to Ref. 12 an increase of P_{Cl} from 0.1 to $1.5 \cdot 10^{-7}$ cm/s in the presence of *t*-butylhydroperoxide is paralleled by an oxidation of 60% of the total hemoglobin. In the secondary damage process described here, after the removal of *t*-butylhydroperoxide, the same increase of leak permeability is only accompanied by an oxidation of additional 7% of hemoglobin.

The characteristics of the leaks forming in cells after pretreatment are similar to those produced in the presence of *t*-butylhydroperoxide [12]. Apparent radii between 1.0 and 1.5 nm can be assigned to the induced 'leak sites'. Lysis induced by uptake of solutes and water via these leak sites is colloid-osmotic and can be suppressed by impermeable solutes (Fig. 5). The apparent number of leak sites per cell is probably lower than 1. This number may be derived from their radii and the measured leak permeabilities, if it is assumed that the leak sites contain bulk water for which normal aqueous diffusion coefficients are valid [12,33]. The previous conclusion [12,30,33] that the defects in damaged cells are dynamic in nature, i.e., fluctuating in time and localisation, is therefore probably also valid for this type of damage.

Similarity between the defects reported here and those produced by other types of oxidative damage [42] is finally demonstrated by the observation that leaks produced by *t*-butylhydroperoxide also serve as flip sites accelerating the transbilayer mobility of long-chain amphipaths (Fig. 7).

Progress of oxidative membrane damage in native, intact erythrocytes merely subjected to a pulse treatment with a chemical oxidant has previously not been observed to our knowledge, although some indications were available [16,43]. Chan and collaborators [44,45] have demonstrated that lipid peroxidation initiated in isolated ghosts by photooxidative or chemical means, will proceed in the presence of traces of transition metals (Cu^{2+} or Fe^{2+}). A comparable phenomenon was reported by Girotti et al. [46] for resealed ghosts which became leaky in the presence of superoxide and ascorbate when lipid hydroperoxides had first been generated by photo-oxidation.

Detectable levels of lipid hydroperoxides do not accumulate in erythrocytes treated with *t*-butylhydroperoxide [12,15]. They are supposed to be decomposed immediately by iron complexes. Moreover, azide and EDTA act inhibitory under the conditions of Chan [44,45] and Girotti et al. [46], while under our conditions azide and cyanide stimulate and EDTA is ineffective. It is therefore unlikely that traces of lipid hydroperoxides, not decomposed during the pre-exposure provide the original cause of the continuing peroxidation after removal of exogenous *t*-BuOOH. The event leading to formation of oxy-radicals remains to be clarified. A similar phenomenon has, however, been described in artificial systems consisting of hemoglobin and unsaturated phospholipids [19].

The damage in the *t*-butylhydroperoxide-pretreated cells most certainly involves an iron-catalyzed Haber-Weiss reaction [35,48]. This view is supported by the inhibitory effect of desferrioxamine, known as a potent suppressor of Fe^{3+}-catalyzed peroxidative reactions [35,48]. A catalytic function of heme-derived, free or complexed iron may thus be postulated. A net oxidation of heme-iron, on the other hand, as required for the activation of *t*-butylhydroperoxide [13–15], is obviously not involved. Evidence for the presence of higher oxidation states of heme-iron in *t*-butylhydroperoxide-treated erythrocytes has been provided [17]. Oxidation of lipids and other compounds in the presence of peroxide-activated methemoglobin or metmyoglobin is a well supported observation [49,50]. The hemichromes attaching to the membrane under our experimental conditions (cf. Fig. 3) may thus play an important role in the develop-

ment of oxidative damage, acting as catalyst but also as terminators of oxidative chain reactions. The whole sequence of injurious events following pretreatment with *t*-butylhydroperoxide thus may be restricted to the membrane in contrast to the situation in the presence of *t*-butylhydroperoxide.

As mentioned above, we consider the stimulation by azide and cyanide to be due to complexation of ferric heme iron. In the azido- or cyano-liganded form such heme-iron complexes are less potent scavengers of alkoxy (and peroxy-?) radicals [12]. The contribution of azidyl radicals [47] does not seem very likely since cyanide, which does not form reactive radicals to our knowledge, binds avidly to ferric heme and stimulates oxidative damage like azide. Whether inhibition of catalase [21] also contributes to the stimulatory effect of azide remains to be studied.

The availability of catalytically active iron is probably not sufficient, however, for the oxidative leak formation in cells pretreated with *t*-butylhydroperoxide. As an additional requirement endogenous protective mechanisms of the cell have to be exhausted. This is indicated by the observation that addition of metabolic substrates (e.g. glucose, inosine) suppresses oxidative damage (Table II). Moreover, depletion of membrane tocopherol during the pretreatment will also contribute to the development of membrane injury (see Ref. 51).

Leak formation in *t*-butylhydroperoxide pretreated cells is suppressed by thiourea at concentrations which have no or very little influence on oxidative degradation of membrane phospholipids, and no effect at all on membrane thiol oxidation and degradation of hemoglobin. These findings clearly demonstrate that the formation of membrane leaks in cells under oxidative stress can at least partly be dissociated from lipid peroxidation. This can also be accomplished by a number of other antioxidants (Deuticke, B., et al., Ref. 60). The basis for this dissociation is not yet clear. One might consider that either different radicals are involved in causing membrane defects and propagation of lipid peroxidation or the same radical species acts at sites differing in reactions conditions or accessibility for antioxidants. Regardless of the mechanistic details, the observation of a selective inhibition of leak formation strongly suggests that peroxidized lipids are not the structures forming the leaks induced by radicals in the erythrocyte membrane.

Concluding remarks

Lasting progress of an oxidative membrane damage only initiated in erythrocytes by short treatment with a peroxide deserves interest also under general aspects: (a) In studies of membrane function in cells following oxidative damage, the progress of this damage during the functional analyses has to be considered. (b) Peroxide-pretreated cells may be an interesting model for similar processes occurring in pathological conditions. It has long been recognized that in sickle cell anemia [52,53] in thalassemia [54], in cells containing other unstable hemoglobins [55–57] but also in erythrocytes in contact with stimulated neutrophils [58] oxidative membrane damage may play an important role. The low level of these alterations has made their investigation tedious. In some cases they are accompanied by precipitation, at the membrane, of hemoglobin in amounts only slightly lower than in cells pulse-treated with *t*-butylhydroperoxide [52]. Mere initiation of oxidative chain reactions by the *t*-butylhydroperoxide avoids progressive damage of cytoplasmic constituents like hemoglobin and allows to set the levels of primary membrane alteration deliberately. This may facilitate the elucidation of the consequences.

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