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Uncoupling of oxidative leak formation from lipid peroxidation in the human erythrocyte membrane by antioxidants and desferrioxamine

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Human erythrocytes, briefly exposed to *t*-butylhydroperoxide and then incubated further in the absence of exogenous oxidant, undergo lipid peroxidation and formation of aqueous membrane leaks. Leak formation can be suppressed by various types of antioxidants and by desferrioxamine at concentrations at which lipid peroxidation still proceeds almost unaltered. This uncoupling of the two manifestations of an oxidative membrane damage indicates that loss of the barrier properties is not an obligatory consequence of the presence of peroxidized lipids in biological membranes.

Oxidative damage of biological membranes has become a topic of increasing biomedical interest [1–3]. Numerous functions of membranes are perturbed when oxygen-derived and other radicals induce peroxidation of lipids [1,2] and oxidative modification of proteins [4,5]. One major functional consequence of this damage is the breakdown of the membrane barrier going along with a dissipation of ion gradients and eventually cell swelling and lysis [5–9]. We are presently studying the molecular basis of oxidative leak formation in erythrocytes treated with organic hydroperoxides [9]. Aqueous leaks (r_{app} 0.6–0.7 nm) are formed in the presence of *t*-butylhydroperoxide [9] but also develop when the cells are only pretreated briefly with the agent, then washed thoroughly and incubated further without any added oxidant [10]. This oxidative alteration may resemble, on a magnified scale, damage observed in red cells in hematological diseases [11–13].

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In butylperoxide-treated erythrocytes increasing leak permeability (P) and progressive peroxidation of polyunsaturated fatty acids are coupled phenomenologically to each other by a relationship $P = 3^{MDA}$ [9]. It is not clear, however, whether leak formation is a direct consequence of peroxidation of acyl chains and the resulting alteration of lipid patterns [7,9,12] and of the physical state of the membrane lipids [14,15]. In artificial lipid membranes leak formation by peroxidizing acyl chains has been demonstrated [16]. In biological membranes direct or indirect alterations of proteins have to be considered as alternatives [4,5]. Experimental uncoupling of leak formation from lipid peroxidation might be helpful to clarify the relationship between the two events and thus to establish the nature of the oxidative leaks. Hemolysis occurring in cells under thiol-induced oxidative stress has been shown to be suppressed by dimethylsulfoxide, while lipid peroxidation still proceeded [17]. Moreover, it was recently reported [8] that the antioxidant, thiourea, dissociates leakiness from lipid peroxidation in the presence of *t*-butylhydroperoxide. We now demonstrate that

rather generally the formation of aqueous leaks can be uncoupled from lipid peroxidation by various antioxidants in erythrocytes exposed to an essentially endogenous oxidative stress during a post-incubation after pulse treatment with *t*-butylhydroperoxide.

Human erythrocytes, from blood stored for less than 5 days, were washed and treated with *t*-butylhydroperoxide (2 mM) in 20 volumes of medium A (90 mM KCl, 40 mM NaCl,12.5 mM phosphate buffer (pH 7.4) 20 mM sodium citrate, 2.5 mM sodium azide). After 15 min incubation at 37 °C the suspension was rapidly cooled to 0–2 °C, centrifuged (10 min, $5000 \times g$) and the cells washed three times with an excess of medium A at 0 °C to remove butylhydroperoxide. These pretreated cells were then incubated for 40 or 60 min at 37 °C in 12 volumes of medium A containing the antioxidants to be tested (dithioerythritol, diethyldithiocarbamate (Sigma); thiourea (Merck, Darmstadt); cysteamine (Koch-Light, Colnbrook, U.K.): mercaptoethanesulfonic acid (UCB, Kerpen); desferrioxamine methosulfate (Desferal®; CIBA). Peroxidative membrane reactions were terminated by addition of butylated hydroxytoluene (100 μM). The cells were washed and tested for manifestations of membrane damage.

Leak permeability, defined as the stilbenedisulfonate-insensitive ^{36}Cl-permeability, was quantified by measuring tracer fluxes as described previously [17]. Progress of lipid peroxidation, taken to be reflected by the formation of thiobarbituric-acid-reactive material * and expressed in terms of malondialdehyde was determined as described in Ref. 9.

In the case of thiourea [10] and desferrioxamine containing systems an interference of the agents with the colour-forming reaction was corrected for on the basis of calibration curves. The other agents used did not interfer.

A 15 min treatment of human erythrocytes with *t*-butylhydroperoxide (2 mM) causes only a slight lipid peroxidation (10.7 nmol malondialdehyde/ml cells) and a minor increase of leak permeability for Cl^- ($P_{Cl} = 5 \cdot 10^{-9}$ cm $\cdot$ s^{-1}) even in the presence of azide which suppresses precipitation of oxidized hemoglobin at the inner membrane surface and amplifies membrane damage by suppressing its radical scavenging properties [9]. When cells pretreated in this way are washed thoroughly and incubated in the absence of exogenous oxidant, progressive and marked lipid peroxidation as well as formation of leaks occur, probably due to the propagation of a chain reaction initiated by the brief pretreatment with *t*-butylhydroperoxide. Both manifestations of oxidative damage (72 nmol malondialdehyde per ml cells. $P_{Cl} = 8 \cdot 10^{-8}$ cm $\cdot$ s^{-1} after 60 min) can be suppressed by various types of protective agents, such as thiol and phenolic antioxidants and the metal ion chelator desferrioxamine. Leak formation is suppressed, however, much more effectively than lipid peroxidation. The ratios between the concentrations of antioxidants required for 50% suppression of leak formation and for 50% suppression of lipid peroxidation (Table I) vary between about 2–4 and 28 or even 51. It is presently not clear why

TABLE I

CONCENTRATIONS REQUIRED FOR 50% SUPPRESSION OF THE DEVELOPMENT OF OXIDATIVE DAMAGE IN ERYTHROCYTES PRETREATED WITH *T*-BUTYLHYDROPEROXIDE

Cells pretreated with *t*-butylhydroperoxide for 15 min at 37 °C were washed and then incubated for 40 or 60 min without exogenous oxidant in the presence of various concentrations of the antioxidants.

	Concentration (mM) for 50% suppression of		Ratio $\frac{I_{50(P)}}{I_{50(L)}}$
	Leak formation $I_{50(L)}$	Peroxidation $I_{50(P)}$	
Thiourea	2.5	≈ 70	28
Dithioerythritol	0.14	0.45	3.2
Mercaptoethanol	0.13	1.1	8.5
Mercaptoethane sulfonate	0.22	0.55	2.5
Cysteamine	0.007	0.36	51
3,4-Dihydroxybenzoate	0.015	0.07	4.7
Diethyldithiocarbamate	0.0012	0.004	3.3
Butylated hydroxytoluene	0.00045	0.0009	2.0
Desferrioxamine	0.40	3.0	7.5

* Progressive lipid peroxidation in cells pulse-treated with *t*-butylhydroperoxide was also ascertained by following the disappearance of polyunsaturated fatty acids (quantified by HPLC, Heller, K.B. and Deuticke, B., unpublished results) as well as of aminophospholipids [10].

cysteamine and thiourea are so much more effective than the other antioxidants.

The 'uncoupling' of leak formation from lipid peroxidation thus obtained is further illustrated in Fig. 1, in which the fractional suppressions of the two manifestations of oxidative damage are plotted against each other for various concentrations of all the agents studied. The data indicate that up to 50% of the leak formation in *t*-butylhydroperoxide-pretreated cells can be inhibited without any suppression of lipid peroxidation, while inhibition exceeding 50% is accompanied by a suppression of lipid peroxidation, too. It seems remarkable, that both, free radical scavenging or reducing antioxidants, and desferrioxamine, the potent chelator of Fe^{3+} ions [19], dissociate leak formation from lipid peroxidation. The selective suppression of leak formation at low antioxidant levels is due to a delay in its onset as could be demonstrated by following the time-course of the development of oxidative damage (data not shown).

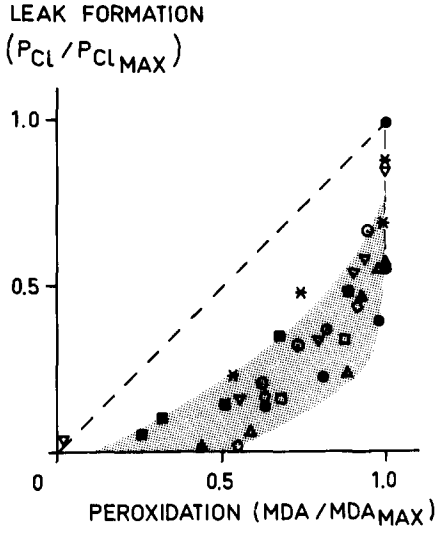

Fig. 1. Uncoupling of peroxidation from leak formation by antioxidants in erythrocytes incubated after pretreatment with *t*-butylhydroperoxide. Peroxidation (MDA/MDA_{max}) and leak formation ($P_{Cl}/P_{Cl_{max}}$) in the presence of antioxidants, normalized to values in their absence. Dashed line: line of unity, expected in the case of tight coupling. ●, Thiourea; ○, cysteamine; ■, 3,4-dihydroxybenzoate; □, mercaptoethane sulfonate; △, mercaptoethanol; ▲, dithioerythritol; ▽, diethyldithiocarbamate; *, desferrioxamine; ⊙, butylated hydroxytoluene. MDA, malondialdehyde.

These observations demonstrate that leakiness – and subsequent lysis – of erythrocytes subjected to oxidative stress is not a direct consequence of the destruction of membrane polyunsaturated fatty acids. Considerable peroxidation of fatty acids can occur without simultaneous alterations of the barrier properties of the membrane. The uncoupling of the two processes is brought about by antioxidants known for their capacity to scavenge hydroxyl, alkoxy- and peroxy-radicals [20–22], but also by complexation of iron.

Since leak formation is not coupled in a fixed relationship to lipid peroxidation, it is also unlikely that decomposition products of peroxidized lipids, e.g. malondialdehyde or 4-hydroxyalkenals, which react with proteins and lipids [23,24], are responsible for leak formation. As an alternative, leak formation can be attributed to oxidative damage of proteins which has already been shown to be the primary event in the formation of aqueous leaks in erythrocytes treated with the SH-oxidizing agents diamide, iodate, and periodate [18,25]. In the present case SH-groups are probably not the target, since their oxidation to disulfide bonds in *t*-butylhydroperoxide pretreated cells is not suppressed by antioxidants [10]. The free radicals causing leak formation via protein damage may not be different from those propagating lipid peroxidation since (a) their formation also requires iron in a form that can be inactivated by desferrioxamine and (b) leak formation is suppressed by the same antioxidants that suppress lipid peroxidation, even at lower concentrations.

According to our results the two manifestations of oxidative damage of the erythrocyte membrane are not arranged in sequence, but reflect parallel events. Both manifestations seem to require free iron, which has recently been shown to be released from hemoglobulin during oxidation by *t*-butylhydroperoxide [26]. The high sensitivity of leak formation, in contrast to lipid peroxidation, to both, an iron chelator and various antioxidants may arise from a better accessibility, to all these agents, of the sites at which an iron-catalyzed radical formation and chain propagation induce leak formation. It would seem likely that these are superficial sites in a more polar environment. It has been demonstrated in artificial systems [27] and in microsomal membranes [28,29] that simul-

taneous manifestations of oxidative injuries promoted by oxygen-derived radicals and iron catalysts may be based on different processes. This is obviously also possible in plasma membranes as suggested by earlier observations in hepatocytes [6] and demonstrated here for the erythrocyte membrane in which peroxidized lipids may be the source of radicals that cause leaks by modifying proteins but are not the structural elements of these leaks.

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