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Injury of rat thymocytes caused by exogenous peroxyl radicals in vitro

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Abstract

The aim of this study was to investigate oxidative cell injury in rat thymocytes under conditions of radical generation exterior to the cell utilizing the thermolabile azocompound 2,2'-azobis(2-amidinopropane) dihydrochloride to generate peroxyl radicals at a constant and reproducible rate. This initiator, being water-soluble and endowed with a positive charge, is suitable for studies on oxidative damage of biomembranes induced in the external water environment. The relationship between cell viability, lipid and thiol oxidation and chain-breaking antioxidant depletion was studied. During the first hour of treatment cell viability decreased slightly, protein sulfhydryl groups were consumed slowly and no significant production of conjugated dienes occurred. After 90 min of incubation, when thymocyte permeability started to increase, the concentration of α-tocopherol decreased gradually, significant changes of polyunsaturated fatty acids occurred and a rapid phase of thiol oxidation commenced. It can be concluded that, under conditions of an exogenous oxidant challenge, initially the cell membrane provides a physical barrier to the entrance of radicals to the thymocyte. When peroxyl radicals gain access to the membrane and the molecular barrier begins to disorganize, the oxidizable cellular components become susceptible to massive attack.

Keywords: Thymocyte; Azothermal initiator; Lipid peroxidation; Thiol oxidation

1. Introduction

Mammalian cells and tissues are continuously exposed to oxidants of both endogenous and exogenous origin. Cells possess antioxidant defences to counteract the effects of free radical generation. Normally, these mechanisms are able to cope with an excess of free radical generation but, when these protective mechanisms fail, cellular damage can occur. A role for free radicals has also been suggested for numerous disfunctions [1], pathologies [2] and, recently, in the pathogenesis of acquired immunodeficiency syndrome [3].

The main mechanism by which activated oxygen species injure cells and tissues remains rather speculative. Many investigators have suggested that the oxidation of polyunsaturated fatty acids in the membrane represents the major pathway by which free radicals mediate their cytotoxic effects [4]. It is also known that free radicals can damage membrane proteins by causing cross linking [5], fragmentation [6] and amino acid modification [7]. Moreover, oxygen radicals and other activated oxygen species react readily with nucleic acids [8,9] and carbohydrates [10,11].

The aim of this study was to investigate the relationships between exogenous free radical-mediated cytotoxicity, lipid and thiol oxidation and chain-breaking antioxidant depletion using rat thymocytes as target cells. We chose thymocytes for the following reasons: (i) T cells may be easily affected by dietary lipid peroxides [12,13]; (ii) T cells are, as any other, subject to variation in oxygen tension and to the attack of different toxic compounds; these conditions may promote the generation of activated oxygen species of free radical character, which may depress the functions of these immunocompetent cells; (iii) T lymphocytes, due to their integral involvement in inflammatory processes, are likely to find themselves in vivo in an oxidative environment, in which products of oxidative processes might play a pathophysiological role; (iv) free thymocytes can be easily isolated from their supporting matrix without affecting the integrity of the cell membrane and without using enzymatic systems (collagenase, trypsin etc.). Thus, the isolation procedure employed results in a

Abbreviations: AAPH, 2,2'-azobis(2-amidinopropane) dihydrochloride; PBS, phosphate-buffered saline; SDS, sodium dodecylsulfate; DTNB, 5,5'-dithio-bis(2-nitrobenzoic acid).

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very high yield of viable cells so that oxidative cell injury can be investigated in intact thymocytes.

We chose the 2,2′-azobis(2-amidinopropane) dihydrochloride as the source of free radicals since, unlike most experimental systems suitable for the induction of oxidative stress, this compound does not require the addition of potentially interfering cofactors and transition metals. Furthermore, this initiator decomposes thermally even at relatively low temperatures and generates free radicals in the water environment at a constant rate. Its use has the advantage of allowing control over both the nature (carbon-centered free radicals that rapidly generate peroxyl radicals via an interaction with molecular oxygen) and the intensity (by varying the chemical's concentration) of the oxidative stress applied to cell samples [14,15]. In the present communication we report data obtained using this free radical generating system with thymocytes, which have allowed for a better understanding of the mechanism of oxidation, particularly when the radical attack originates in the milieu external to the cell.

2. Materials and methods

2.1. Chemicals and biochemicals

2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) was purchased from Polysciences (Warrington, PA) and stored as a 0.4 M solution in phosphate-buffered saline (PBS) at −20°C in the dark. Sodium dodecylsulfate (SDS), α-tocopherol and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were obtained from Sigma (St. Louis, MO). All aqueous solutions were passed through Chelex-100. All other chemicals used were of highest available purity grade and were purchased from Merck (Darmstadt, Germany) or Sigma (St. Louis, MO). Solvents were HPLC or spectral grade.

2.2. Thymocyte preparation

Young male Wistar rats (2–4 months) were killed by decapitation and thymus glands were rapidly removed, washed in PBS, divided into small pieces, filtered through nylon gauze and thymocytes obtained by centrifugation at $800 \times g$ for 5 min. The pelleted cells were suspended in PBS, counted and diluted to 10^8 cells/ml. In some preparations, where erythrocytes were present, the pelleted cells were suspended in 0.8% (w/v) NH_4Cl in 10 mM Mops, pH 6.5, and maintained for 10 min at 37°C to lyse the red blood cells [16]. The protein content of the thymocyte suspension was estimated [17] using crystalline bovine serum albumin as standard.

2.3. Experimental protocol

Initiating radicals were generated by the thermal decomposition of the azocompound: the reaction mixtures containing the cell suspension ($20 \cdot 10^6$ cell/ml) and different concentrations of AAPH (10–50 mM) were incubated at 37°C. At different time intervals, aliquots of the reaction mixtures were centrifuged at $800 \times g$ for 10 min to remove the azocompound. Appropriate blanks lacking the thermal initiator were also prepared. Pelleted cells were suspended in 1–2 ml of PBS, and were employed for the several determinations.

2.4. Cytotoxic damage evaluation

Cell viability was assessed by means of the Trypan blue exclusion test. Dye-negative and -positive cells were counted in a Burker hemocytometer. The viable cell percentage of the total cell number was referred to as 'percent cell viability'. Cell lysis was evaluated by determining the total cell number.

2.5. Evaluation of lipid peroxidation

Aliquots of the reaction mixtures ($(400–600) \cdot 10^6$ cells) were withdrawn at different time intervals, centrifuged and suspended in 2 ml of PBS. Total lipids were extracted according to Bligh and Dyer [18]. Lipid peroxidation was assessed by measuring conjugated and ketone diene formation and polyunsaturated fatty acid changes. Conjugated dienes and ketone dienes were quantified in cyclohexane (about 0.5 mg lipid/ml) at 233 nm ($\epsilon = 28\,000$ M^{-1} cm^{-1}) and 275 nm ($\epsilon = 20\,000$ M^{-1} cm^{-1}), respectively [19]. Spectra were recorded at 350–200 nm against a blank containing the same amounts of lipids lacking the initiator and maintained under the same experimental conditions. The fatty acid composition of the lipid extracts at different time intervals was determined by gas chromatography after transesterification [20] using a Carlo Erba HRGC 4160 model (Milano, Italy) equipped with a flame ionization detector and a fused silica capillary column (0.25 mm × 25 m, film of cyanopropylsilicone). The oven temperature was programmed from 140°C to 250°C at 5°C/min and the detector temperature was maintained at 290°C. Peaks were identified using standard fatty acid methyl esters from Supelco (Bellafonte, PA, USA).

2.6. α-Tocopherol determination

α-Tocopherol was determined in total lipid extracts by HPLC utilizing a LiChrosob Si-60, 5 μm column (25 × 0.4 cm i.d.) using fluorescence detection [21]. α-Tocopherol in n-heptane was used as an external standard. The elution was isocratic at a flow rate 1.5 ml/min with 0.8% isopropanol in hexane.

2.7. Protein thiols and reduced glutathione determination

Protein sulfhydryl groups were measured according to Di Monte [22]. Aliquots of the reaction mixtures ($20 \cdot 10^6$ cells) were centrifuged Pelleted cells were suspended in

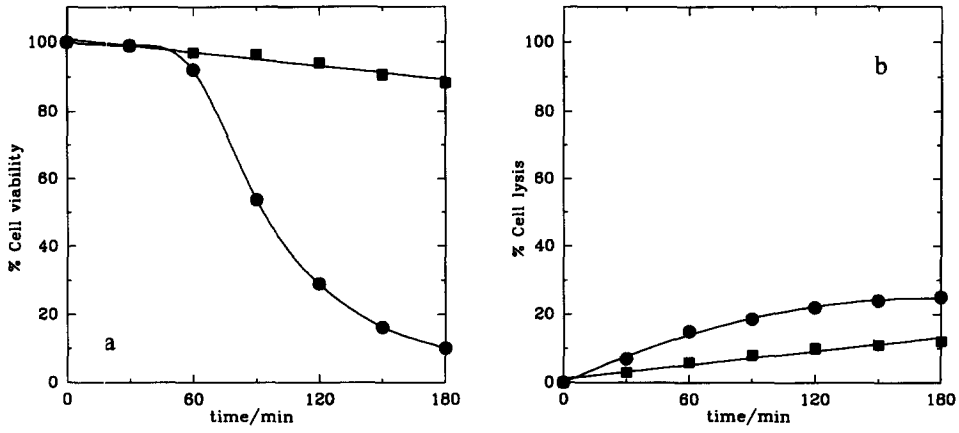

Fig. 1. Effect of AAPH on cell viability and lysis of rat thymocytes. Thymocytes ($20 \cdot 10^6$ cells/ml) were incubated at 37°C in PBS, in the presence (●) and absence (■) of 25 mM AAPH. Cell viability (a) was estimated by Trypan blue exclusion and cell lysis (b) by counting the total cell number. The values are means of five independent determinations.

PBS and 6.5% (w/v) trichloracetic acid was added. Supernatants were used for GSH determination by using the colorimetric assay of Harlan et al. [23]. Pellets were solubilized in 8% (w/v) SDS for 1–2 h and suspended in 0.5 M Tris-HCl, 5 mM EDTA, pH 7.6. The reaction was started by adding 2 mM DTNB (160 μM final concentration) and the increase of absorbance was followed at 412 nm until a constant value was obtained (about 5 min). The actual concentration of sulfhydryl groups was calculated by using an $\epsilon = 13\,600$.

3. Results

3.1. Effect of AAPH on viability and lipid peroxidation of thymocytes

Initial observations were on cell viability determined by means of the Trypan blue exclusion test during the generation of exogenous oxygen-centered radicals by the water-soluble AAPH. Thymocytes were incubated at 37°C either in the presence of different concentrations of AAPH or in its absence and aliquots removed for assay after several periods of incubation. The viability and the lysis of cells without AAPH treatment were affected by only 10% during incubation periods of up 3 h in PBS (Fig. 1). The presence of 25 mM AAPH, the concentration used in the subsequent experiments, allowed measurements to be performed on suspensions containing a majority of non disrupted cells. Under these conditions, in fact, thymocyte viability decreased slightly during the first hour of incubation and then rapidly to 25% after 2 h (Fig. 1a) and the number of cells decreased only by 20% during incubation periods of up 3 h in PBS (Fig. 1b).

Oxidant stress in biomembranes is known to induced peroxidation of membrane lipids with the potential for the decomposition of membrane lipids and injury to the membrane structure. Thus, we assessed lipid peroxidation by

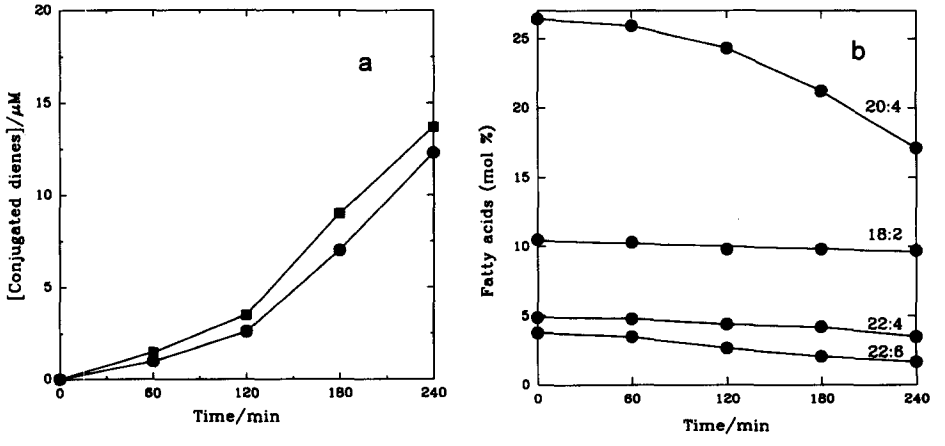

Fig. 2. Time-course of conjugated and ketone diene formation and polyunsaturated fatty acid degradation in thymocyte lipids induced by AAPH. Thymocytes ($20 \cdot 10^6$ cells/ml) were treated with 25 mM AAPH at 37°C in PBS. (a) Conjugated diene (■) and ketone diene (●) formation was quantitated in cyclohexane at 233 nm and 275 nm, respectively, against a blank containing the same amount of lipids. (b) Composition of polyunsaturated fatty acids at different time intervals is expressed as mol%. The values are means ± S.D. of three independent determinations.

Table 1
Fatty acid composition of rat thymocyte lipids.

Fatty acid	Composition
16:0	24.8±1.5
16:1	1.8±0.1
18:0	16.0±1.2
18:1	11.7±0.9
18:2	10.5±0.7
20:4($n-6$)	26.4±1.7
22:4($n-6$)	4.9±0.5
22:6($n-3$)	3.8±0.2
U.I. [a]	169

Composition is expressed as mol% and represents the means±S.D. of three determinations.
[a] The unsaturation index (U.I.) is defined by $\Sigma m_i \cdot n_i$, where m_i is the percentage and n_i the number of double bonds, taking into account all fatty acids with two or more double bonds.

measurement of both the first observable peroxidative product, i.e., conjugated dienes, and the changes of oxidizable substrate, i.e., polyunsaturated fatty acids (Fig. 2).

The spectrum of peroxidized lipids is characterized by an intense band near 233 nm, with a lesser secondary maximum due to ketone dienes in the 260–280 nm region [24]. Both the absorbance at 233 and 275 nm remained practically unchanged during the first hour of incubation and began to increase during the second hour in AAPH-treated thymocytes; subsequently, absorbance values and the correspondent amounts of dienes were found to increase to a greater extent (Fig. 2a). Similar results were also obtained by measuring the fatty acid changes in the presence of AAPH (Fig. 2b). The fatty acid composition of total lipids extracted at 0 time from thymocyte preparations is shown in Table 1.

Linoleic and arachidonic acids had the highest concentrations among the polyunsaturated fatty acids. The unsaturation index was found to be 169. As reported in Fig. 2b, incubation of T-cells with the azocompound resulted in the degradation of the polyunsaturated fatty acids, which started after 1 h of incubation. The most sensitive fatty acids to peroxidation were arachidonic, docosatetraenoic and docosahexaenoic acids, which decreased by 35%, 28% and 56% of their corresponding control values after 4 h. The decrease in linoleic acid was about one fifth of the decrease in arachidonic acid and the unsaturation index of lipid extract had decreased to 99% (U.I. = 167) of its control value after 1 h of incubation and down to 70% (U.I. = 118) after 4 h. As expected, the level of decomposed fatty acids corresponded to the degree of unsaturation, suggesting that the degradation of the fatty acids was determined by the number of active double allylic hydrogens. These observations are in agreement with a recent paper of Wagner et al. [25] that clearly demonstrates, for the first time on quantitative basis, that the number of bis-allylic positions contained in the cellular lipids of intact cells determines their susceptibility to free radical-mediated peroxidative events. Results reported in Fig. 2 show, therefore, that the exposure to 25 mM AAPH failed to induce significant production of conjugated dienes and significant decrease of peroxidizable substrates in thymocytes during the first hour, but was with significant effect thereafter.

3.2. Changes in the concentration of α-tocopherol in AAPH-treated thymocytes

We hypothesized that the lag phase, observed in the peroxidation of thymocytes treated with 25 mM AAPH, was due to inhibitors of the free radical-mediated chain oxidation of lipids present in the plasma membrane of T-cells. Lipid oxidation, in fact, can be suppressed either by inhibiting the initiation step or accelerating the termination step. In the absence of aqueous enzymatic and nonenzymatic antioxidants, as in this case, the most probable candidates for this antioxidant activity are tocopherol, a potent membrane chain-breaking antioxidant, and surface thiols of membrane proteins, which have been suggested to contribute to the antioxidant capacity [26]. Thus, we measured the rate of α-tocopherol consumption in thymocytes and, unexpectedly, we found that the concentration of this antioxidant remained essentially unchanged for 90 min after the addition of 25 mM AAPH, after which it decreased linearly; however, 50% of α-tocopherol was still present at the end of incubation (Fig. 3). The content of α-tocopherol in control thymocytes showed a slight tendency to decrease during incubation without AAPH.

3.3. Changes in the level of thiol groups in AAPH-treated thymocytes

We then investigated whether the system generating free radical outside of the cell could affect glutathione

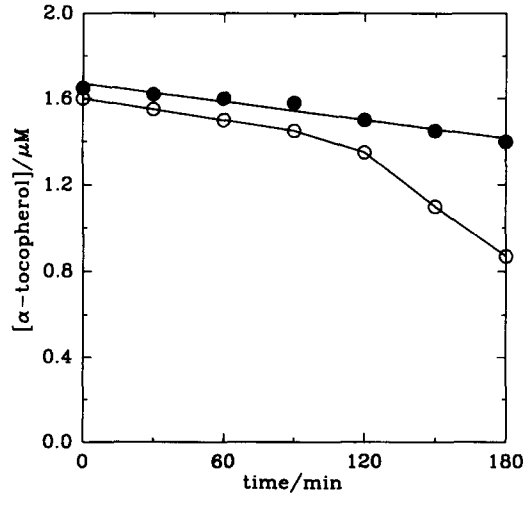

Fig. 3. Consumption of α-tocopherol during the oxidation of rat thymocytes. Thymocytes were incubated at 37°C in PBS in the presence (○) and absence (●) of 25 mM AAPH. The concentration of α-tocopherol was determined by HPLC, as described in Section 2. The values are means±S.D. of three independent determinations.

status and protein sulfhydryl groups of thymocytes. As shown in Fig. 4a, GSH content of both control and AAPH-treated cells decreased in parallel during the first 30 min of incubation, then a higher rate of GSH oxidation occurred in AAPH-treated thymocytes.

The disappearance of protein thiols in control cells and in those subject to oxidation was also followed. The data plotted in Fig. 4b show that, after about 2 h of AAPH treatment in which sulfhydryl groups were consumed slowly, the rate of oxidation increased. It should be noted that the fast phase of thiol oxidation commenced when polyunsaturated fatty acids were degraded and α-tocopherol began to be consumed. These data suggest that peroxyl radicals gained access to intact cell membranes with difficulty during the first two hours of incubation. A confirmation of this hypothesis was obtained by measuring how long AAPH radicals were not accessible to the aqueous compartment of large unilamellar vesicles of dimyristoylphosphatidylcholine in which a hydrophilic paramagnetic probe, such as TEMPOcholine was incorporated. The EPR signal of the nitroxide was stable for two hours both in the presence and absence of AAPH (unpublished data). A further confirmation was provided by some preparations where erythrocytes were present. In this case, thymocytes were treated with 0.8% ammonium chloride in order to lyse the red blood cells. This treatment, followed by incubation with 25 mM AAPH, changed completely the course of oxidation both of GSH and of protein thiols, as shown in Fig. 4. Under these conditions, in fact, AAPH promoted a linear and extensive oxidation of SH: GSH was totally consumed after 90 min of incubation (Fig. 4a) and only 20% of protein sulfhydryl groups were present after 2 h, while in thymocytes not pre-treated with NH_4Cl, 70% of protein thiols were still intact at 2 h of incubation (Fig. 4b).

4. Discussion

The purpose of this research was to obtain information regarding the extent and type of oxidative injury to intact thymocytes under conditions where free radicals are generated exterior to the cell. AAPH has been extensively used in kinetic studies of lipoperoxidation in liposomes [27–29], LDL [30] and erythrocytes [31]. This hydrophilic initiator has been recently used to study the antioxidant role of reduced coenzyme Q homologues in nucleated cells, i.e., isolated hepatocytes [32,33]. The viability of these cells in the presence of 50 mM AAPH decreased rapidly only after 3 h of AAPH treatment, while thymocyte viability decreased to 25% after 2 h of incubation in the presence of 25 mM AAPH (cf. Fig. 1a). This clear difference might be ascribed both to a diverse balance in the prooxidant/antioxidant steady state of the two cell types and, more probably, to the fact that thymocytes were incubated in PBS and hepatocytes in Eagle's MEM. In this last condition peroxyl radicals formed by the azoderivative were available also for medium components, thus the whole radical attack was decreased in the case of hepatocytes. It can be concluded that caution has to be used when the effect of AAPH concentration on cell viability and oxidation parameters is compared in different cell types unless the same medium is used. This may be valid also for other radical initiators.

Free radicals attacking biomembranes can lead to the oxidative destruction of polyunsaturated fatty acid residues of phospholipids and eventually cause membrane damage followed by cell lysis. It can be said, from the results shown in Fig. 2, that peroxyl radicals generated from the decomposition of AAPH did not induce significant peroxidation of whole cellular lipids after 60 min of treatment and, at the same time, α-tocopherol was spared (cf. Fig.

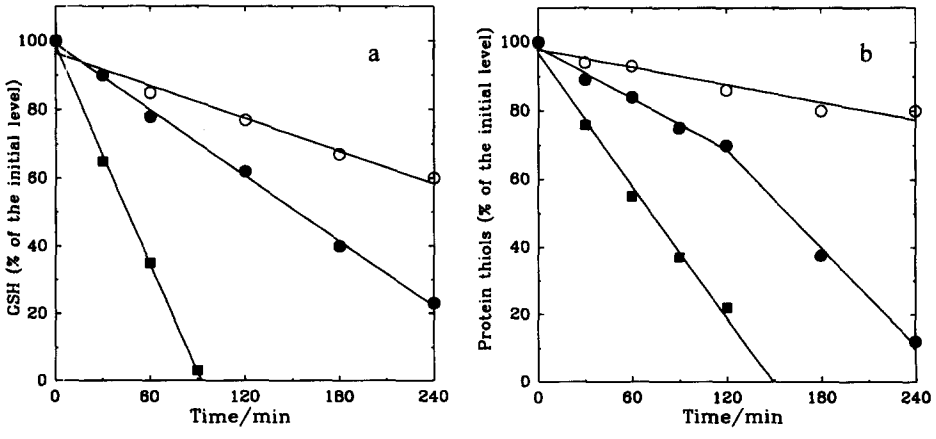

Fig. 4. Effect of AAPH on the oxidation of glutathione and protein sulphydryl groups of rat thymocytes. The decrease of glutathione (a) and protein thiols (b) is expressed as percentage of 0 h level. Thymocytes were incubated at 37°C in PBS in the presence (●) and absence (○) of 25 mM AAPH. Thymocytes pre-treated with 0.8% NH_4Cl were incubated in the presence of 25 mM AAPH (■) as described in Section 2. The initial values of GSH and protein thiols were 20 ± 3 and 65 ± 10 nmol/mg protein, respectively. The values are means ± S.D. of three independent preparations.

3). After 90 min, when thymocyte permeability increased, these oxidizable substrates were attacked and the concentration of α-tocopherol began to decrease gradually. However, the changes of polyunsaturated fatty acids were not massive even after 3 h and the correspondent decrease of α-tocopherol was 50% at the end of incubation. These data on lipid peroxidation may account for the low number of lysed cells, which did not surpass 20% of the initial cell number after 3 h of AAPH treatment (cf. Fig. 1b).

In the experiments performed to evaluate sulfhydryl oxidation in intact thymocytes exposed to exogenous oxygen radicals, we observed that GSH depletion was constant and fast (cf. Fig. 4a), while the oxidation rate of protein thiol groups occurred via successive slow and fast periods (cf. Fig. 4b). We suggest that this reaction was initially slow for the following reasons: (i) the peroxyl radical generator is endowed with a positive charge that could cause a partial lack of accessibility of AAPH in intact cells; (ii) the reaction could be hindered due to steric factors at the membrane surface and therefore, the structure of the thymocyte membrane was per se effective in physical protection against aqueous peroxyl radicals; (iii) GSH protected protein thiols against loss during thymocyte peroxidation. The physiological relevance of GSH protection of the exofacial membrane SH population is supported by Reglinski et al. [34] and Shandu et al. [35]. With respect to the first two suggestions, when thymocytes were treated with 0.8% NH_4Cl in order to lyse erythrocytes a likely permeabilization of T cells occurred, which favoured the access of AAPH molecules to thiols of membrane and intracellular proteins and significantly changed the time course of sulfhydryl oxidation. In conclusion, the overall data support the view that, under conditions of AAPH challenge, thymocyte membranes physically protect the cell, but when aqueous peroxyl radicals gain access to the membrane, the molecular barrier begins to disorganize with consequent massive thiol oxidation, lipid peroxidation and concomitant consumption of α-tocopherol.

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