Current Topics in Biophysics, vol. 18(2), 119-121

FREE BASE AND METALLOPORPHYRIN RADICALS

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(Accepted 28 December 1994)

In this study we have synthesized meso-tetra(4-hydroxy-3-methoxy)phenylporphyrin and (4-methoxy)phenylporphyrin as free bases and in a metal Ni^{2+}-liganded form. Electron paramagnetic resonance spectra of porphyrins in a powder state were measured at room temperature. Two of the four parallel Ni lines with nitrogen superhyperfine structure are well resolved. The third metal parallel line is overlapped by the stronger perpendicular lines to different extents, whereas the fourth parallel Ni line is invariably completely overlapped. The central group of strong lines, together with the strong free radical line, results mainly from the perpendicular feature.

INTRODUCTION

The study of metalloporphyrins by electron paramagnetic resonance (EPR) dates back to 1954 when Ingram (Swartz, Bolton & Borg, 1972) first detected resonance in some derivatives of chlorophyll, hemoglobin and phthalocyanine. Since then, much work has been done and some progress in the understanding of their electronic structure has been achieved.

Interest in metalloporphyrins probably originated from the biological implications, but these compounds are chemically important on their own. Their complexes have a square-planar (D_{4h}) or, in some cases, squarepyramidal (C_{4v}) structures, but are unique in that the ligand is a fairly rigid cyclic tetradentate and the metal-ligand distances therefore are very much constrained.

The chemically important electrons of the porphinato ligand are those belonging to the conjugated π-electron system. They occupy orbitals that transform like A_{1u}, A_{2u}, B_{1u}, B_{2u}, and E_g representations of D_{4h} (Symons, 1987). Only the e_g orbitals can bond with d orbitals (d_{xz}, d_{yz}) of the metal, but here the overlap is small. Thust, there are two somewhat isolated electronic systems, one belonging to the metal and the other to the ligand. As a consequence, it is possible to point out that it has been possible in almost all cases to assign either a metal or a ligand reaction to a given oxidation or reduction step of a metalloporphyrin.

In an electronic spectrum of a metalloporphyrin, the very strong π-π^* transitions of the ligand electrons completely mask the much weaker d-d transitions of the metal. For this reason, the usually powerful tool of electron spectroscopy becomes useless in studying the metal part of the

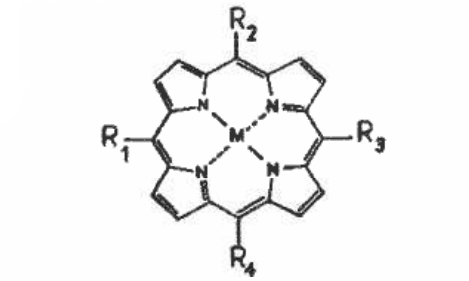

Fig 1. Structure of metalloporphyrin. M-metal ion of Ni^{2+}, Co^{2+} or free base $2H^+$. (a) T4H3MXPP, (b) TMXPP.

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Abbreviations used:
H_2TMXPP – meso-tetra(4-methoxy) phenylporphyrin free-base.
H_2T4H3MXPP – meso-tetra(4 hydroxy-3-methoxy) phenylporphyrin free-base.
Ni- or CoTMXPP – metal-ligated meso-tetra(4-methoxy) phenylporphyrin.
Ni- or CoT4H3MXPP – metal-ligated meso-tetra(4 hydroxy-3-methoxy) phenylporphyrin.

electronic structure. This leaves EPR as the only alternative for such investigations, and indeed, the primary objective of the EPR studies has been the understanding of the part of the electronic structure belonging to the metal.

EXPERIMENTAL

Porphyrins as free bases were synthesized according to our modification of the procedure described by Adler *et al.* (Adler, Longo, Finarelli, Goldmacher, Assour & Korsakoff 1967). Metal porphyrins were obtained using the method of Johnson and Dolphin (1980). Typical procedures were as follows.

Preparation of [5,10,15,20-tetra(4-methoxyphenyl)]-21H,23H-porphine

Propionic acid (80 ml, Fluka) was magnetically stirred and boiled in 250 ml three-necked round bottom flask under reflex condenser. From two funnels p-anisaldehyde (2.72 g, 0.02 mole) in 30 ml of propionic, and pyrrol (1.34 g, 0.02 mole) in 10 ml of propionic acid was simultaneously dropped in during half an hour. After completing aldehyde and pyrrol addition, the mixture was refluxed additional half an hour. Propionic acid was removed under vacuum, and the residue was twice chromatographed on column using Florosil (Fluka) with methylene chloride. The liquor was concentrated to yield crystals to give a total of 0.71 g pure product (21% yield). The obtained product is characterized by following absorption maxima in ethanol solution (in nm): 450, 514, 552, 590, 638, 687.

Preparation of [5,10,15,20-tetra(4-methoxyphenyl)]-21H,23H-porphinato(2-)]-nickel(II)

[5,10,15,20-Tetra(4-methoxyphenyl)]-21H, 23H-porphine (0.136 g, 0.2 milimole) and 0.88 g Ni(II) acetate was dissolved in 50 ml of propionic acid and refluxed for six hours. The mixture was allowed to stand overnight and was then filtered. The product was washed with propionic acid, twice with ether, and than further with water. The purple crystalline product was dried under vacuum to give 0.118 g (68% yield). Chemical structure of the synthesized porphyrins is shown in Fig.1.

EPR spectra were measured at room temperature using Radiopan SE/X-25 spectrometer with RCX 660 microwave cavity (Manikowski, Brzeziński & Olejnik, 1994).

RESULTS AND DISCUSSION

A typical polycrystalline spectra for nickel porphyrins are shown in Fig.2 and 3. Two of the four parallel Ni lines with nitrogen superhyperfine (shf) structure are well resolved and can be used for the accurate determination of $g_{\|}$, $A_{\|}^{Ni}$ and $A_{\perp}^{N}$. The third parallel Ni line is overlapped by the much stronger perpendicular lines to different extents, whereas the fourth Ni parallel line is invariably completely overlapped. The central group of strong lines, to the right of the very strong free radical line, results mainly from the perpendicular feature. The group of strong lines on the very high field end results from the "anomalous line". When small width for the field modulation such as 0.1mT was applied, some of the parallel hyperfine (hf) lines were found to split into at least seven lines spaced with separations of 0.25-0.3mT (Fig.2 and 3). Splitting of this type may be inter-

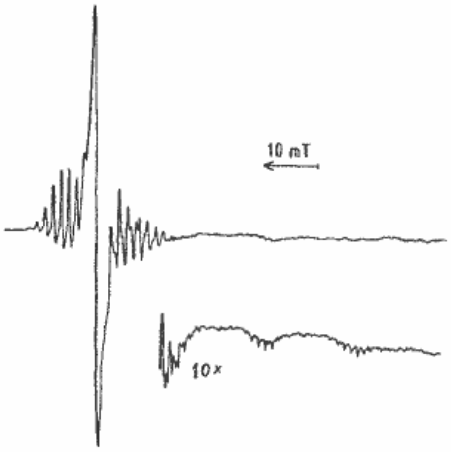

Fig.2. EPR spectrum of NiTMXPP in polycrystalic state. Strongest line belong to free radical of porphyrin ring and is at g = 2.0026. Microwave power attenuation 8 dB, frequency 9411 MHz, modulation amplitude 0.2 mT. Room temperature.

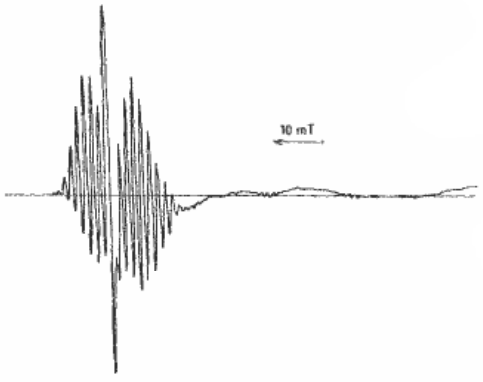

Fig.3. EPR spectrum of NiT4H3MXPP in polycrystallic state. Strongest line belong to free radical of porphyrin ring and is at g = 2.0026. Microwave power attenuation 8 dB, frequency 9408 MHz, modulation amplitude 0.2 mT. Room temperature.

preted qualitatively if the unpaired electron of the nickel interacts with the ligand-nitrogen nuclei. If the four nitrogen ligands are magnetically equivalent, nine shf lines should be observed with relative intensities of 1:4:10:16:19:16:10:4:1. The observed shf structure is consistent with this expectation, if two weakest lines are assumed to be smeared out. The spin density around the nitrogen ligands of NiTMXPP and NiT4H3MXPP is expected to arise from the delocalization of in-plane π bonding or from the configuration interaction of such orbital as in-plane s bonding. To discuss the bonding scheme along the line accurate data for $A_{\parallel}^{N}$ are at least required. Unfortunately we were unable to detect $A_{\parallel}^{N}$.

Acknowledgements

This study was financially supported by Poznań University of Technology.

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Current Topics in Biophysics, vol. 18(2), 122-125

RESPIRATORY BURST OF THE NEUTROPHILS IN ISCHEMIC HEART DISEASE PATIENTS

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(Accepted 28 December 1994)

We measured the oxidative burst of neutrophils obtained from peripheral blood (PB) and coronary sinus blood (CSB) in 28 patients suffering from ischemic heart disease (IHD) with symptoms of unstable angina. The examinations were performed during coronary angiography (CA) or angioplasty (PTCA). We tested the gradient sedimentation-isolated neutrophils using the chemiluminescence (CL) generation system. A mean of the CL (mV · min.) of nonstimulated and PMA and fMLP stimulated neutrophils in IHD patients did not differ significantly from the CL in 15 healthy volunteers which comprised a control group. The oxidative burst of opsonized and nonopsonized zymosan – stimulated neutrophils were significantly ($p < 0.05$) lowered as compared with controls, (CL values: 10202 ± 6270 and 1980 ± 1694 as compared to 15044 ± 6906 and 3562 ± 2554, respectively). There were no differences in the oxidative burst capacity of the PB neutrophils in IHD patients as compared with CSB neutrophils. In the light of our earlier results on the increased CL activity of the cells, measured in whole blood in IHD patients, we hypothesize that the reduction of the CL of isolated neutrophils in the patients may be a result of the isolation and purification procedures. We suggest that the simultaneous measuring of the CL both in whole blood and in isolated neutrophils can answer more questions on the oxidative potential of the neutrophils.

INTRODUCTION

Neutrophils have been implicated in myocardial injury following reperfusion of the ischemic myocardium (Simpiniak & Ozawa, 1993). Besides proteases, oxygen-derived free radicals (OR) are thought to play an important role in the injury (Reimer, Tanaka, Murry, Richard & Jennings, 1990). Neutrophils have been recognized as a main source of the toxic OR produced due to ischemia induced stimuli (Ricevutti, Mazzone, Pasotti, De Servi & Specchia, 1991). Neutrophil derived OR reduce releasing of EDRF and prostacyclin synthesis by endothelial cells in ischemic myocardium (Rubanyi, 1988; Gryglewski, Palmer & Moncada, 1986). This may be a reason of the generation and development of the ischemic changes in the heart. There are many data in the literature documenting increased neutrophil function in patients with IHD (Mehta, Nichols & Metha, 1988). High leukocyte number and activation of the neutrophils have been established as the accessory risk factor of ischemic heart disease (Ernst, Hammersmith, Bagge, Matrai & Dormandy, 1987). Thus, neutrophil activation and OR release are both a cause and a result of ischemic changes in the heart. One of the most sensitive methods of the measuring of neutrophil activation is detection of its luminol-enhanced chemiluminescence capacity during spontaneous and stimulated oxidative burst (Wymann, Von Tscharner, Deranleau & Baggiolini, 1987). The aim of our study was to measure the chemiluminescence activity of neutrophils isolated from peripheral blood and from coronary sinus blood in patients suffering from unstable ischemic heart disease.

METHODS

Patients

We examined 28 patients (20 men, 8 women, aged 48 ± 7.4 years) with clinically proven IHD and with clinical symptoms of unstable angina. They had no antiinflammatory or immunomodulatory treatment 2 weeks before examination. Patients treated with calcium channel blockers or

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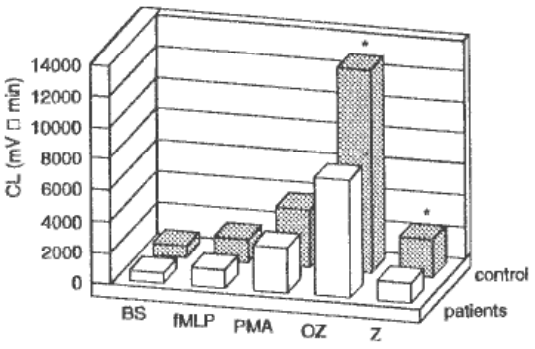

Fig. 1. Chemiluminescence of PB neutrophils.7

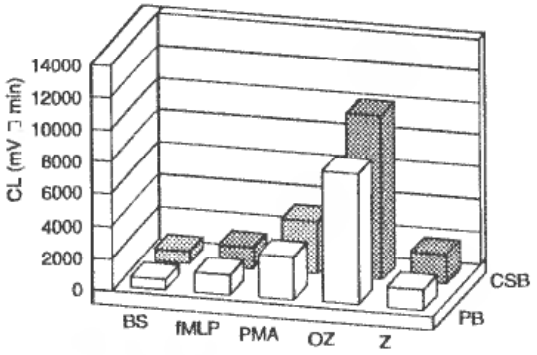

Fig. 2. Chemiluminescence of PB and CSB PMNs.

with a history of myocardial infarction or systemic diseases were excluded.

Fifteen healthy volunteers (12 men, 3 women, aged 44.6 ± 8.6 years) constituted the control group.

Isolation of neutrophils

Blood samples from peripheral vein were taken from healthy controls and IHD patients by vein puncture in the morning hours before breakfast. Blood samples from coronary sinus were taken at the same time point, before PTCA or CA, using catheters inserted into the femoral vein and positioned in the coronary sinus in the right atrium. 15,000 units of heparin was given to each IHD patient at the beginning of the procedure. Blood samples from IHD patients and controls were stored at room temperature before testing and the CL measurements were performed in up to two hours time after sampling the blood.

Neutrophils were isolated from the heparinized blood by one step Gradisol-G (Polfa, Kutno, Poland) gradient sedimentation. Obtained neutrophils were washed twice and suspended at a concentration of $1 \cdot 10^6$ cells per ml in RPMI 1640 (Flow Laboratories, G.B.) culture medium supplemented with 2 mM L-glutamine (Flow Laboratories), 100 U/ml penicillin and 100 µg/ml streptomycin and 10 % of heat-inactivated fetal calf serum (FCS, Gibko) and then used in the test. The same conditions and procedures were maintained in isolation and CL testing of peripheral and coronary sinus blood neutrophils.

Measurement of chemiluminescence

A 1251 luminometer (Bio-Orbit, Turku, Finland) coupled with an IBM-PC AT-compatible computer was used for the measurement of CL. To determine optimum concentration of the stimuli chemotactic peptide – N-formyl-methionyl-leucyl-phenylalanine – (fMLP), phorbol myristate acetate – (PMA) and zymosan (Z) curves were used and dose-effect dependencies were measured. The optimum concentrations of stimuli were used in the measurements: fMLP – 1 nmol/ml, PMA – 200 ng/ml, Z – 0.3 mg/ml. Investigation were carried out at 37°C. A sample contained $2 \cdot 10^5$ neutrophils in 200 µl of PBS, 20 µl of luminol and 10 µl of fMLP or 20 µl of PMA or 30 µl of Z, and PBS to a final volume of 1 ml. Response of the nonstimulated, resting cells (NS) was also investigated. Each measurement was made in triplicate and expressed in mV·min. as the area under the obtained curve of CL versus time (integrated light emission 0–30 min.).

Statistical analysis

Values are expressed as mean ± SD. The Student's t test was applied for the analysis of differences between the data for IHD patients and for healthy controls. Significances of differences between the CL of the neutrophils in peripheral blood and in coronary sinus were obtained applying Wilcoxson matched pair test. The probability value $p < 0.05$ was considered significant.

RESULTS

Values of spontaneous and stimulated CL in IHD patients were lowered as compared to these values in healthy controls. Significant differences were observed in CL stimulated with opsonised and nonopsonised zymosan, respective values being 10202 ± 6270 and 1980 ± 1692 compared to 15044 ± 6906 and 3562 ± 2554 mV·min., Fig 1.

Neutrophils isolated from peripheral blood and from coronary sinus blood in IHD patients had the same respiratory burst capacity, Fig 2.

DISCUSSION

Patients suffering from unstable ischemic heart disease complain to a repeated chest pain as a symptom of transitory myocardial ischemia. Such

short time ischemia results in the release of the inflammatory mediators and cytokines which are potent neutrophil stimuli (Shalaby, Aggraval, Rinderknecht, Svedersky, Finkle & Palladino, 1985; Smith, Sam, Justen, Leach & Epps, 1987; Simon, Chambers, Buchter & Sklar, 1992). Some of these substances act as a priming factor leading to a significant increase in the neutrophil chemiluminescence response to another stimulus (Wiedermann, Niedermuhlbichler & Braunsteiner, 1992). Activation of neutrophils in patients with stable angina has gained experimental and clinical support (Mehta *et al.*, 1988). Increased formation of neutrophil proteases, lipid moieties and OR was demonstrated (Mehta, Dinerman, Metha, Sladeen, Lawson, Donelly & Walin, 1989; Sisson, Prescott, McIntyre & Zimmerman, 1987; Ricevutti *et al.*, 1991). Significant increase in OR generation during myocardial ischemia and immediately after the reperfusion was observed in animal model using electron spin resonance spectrometry (Kobayashi, Watanabe, Ozawa, Hayashi & Yamazaki, 1989). The observed increase in neutrophil OR generation has usually not been directly measured but results from the lower ability of the cells to respiratory burst (De Servi, Mazzone, Ricevuti, Fioravanti, Braunucci, Angoli, Stefano & Specchia, 1990). This result is consistent with our presented findings. The most powerful stimulus of the neutrophil CL is opsonised and nonopsonised zymosan (Cain, Newman & Ross, 1987). The low CL response to stimuli suggest "exhausted" oxidative potential of the cytokine-stimulated neutrophils in ischemic myocardium. Nevertheless, there were no differences between CL activity in peripheral and coronary sinus blood neutrophils. Thus, the flow of the neutrophils through the ischemic myocardium did not result in a significantly higher stimulation of the cells. The presented results disagree with our previous findings on the neutrophil CL measured in whole blood (Baj, Kowalski, Kantorski, Pokoca, Kośmider, Pawlicki & Tchórzewski, 1994). We hypothesize that the long procedure of the cell isolation may lead to the respiratory burst already during the processing of the cells. Thus, the hypersensitivity rather then insensitivity of the neutrophils to stimuli may be a reason of the observed drop in the oxidative potential.

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PARAQUAT-INDUCED CHANGES IN THE STRUCTURE OF ERYTHROCYTE MEMBRANES ARE NOT CAUSED BY LIPID PEROXIDATION

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(Accepted 28 December 1994)

The alterations of the structural properties of human red blood cells upon paraquat treatment were studied using spin labeling method. The increase in membrane lipid fluidity concomitant with the increasing concentrations of paraquat was indicated by the spectra of methyl 12-doxylstearate spin label. However, the elevation in the thiobarbituric reactive substances was not observed. In turn, the spectra of maleimide spin label (4-maleimido-2,2,6,6-tetramethyl-piperidine-1-oxyl) indicated a modification in membrane proteins in isolated erythrocyte ghosts but not in the red blood cells internal proteins. These results suggest that changes in membrane lipid fluidity did not result from lipid peroxidation but rather from the alterations in lipid-protein interactions.

INTRODUCTION

Paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride, methyl viologen, PQ) is a nonselective contact herbicide used in a variety of crops. It exhibits high toxicity for man and animals. Accidentally or intentionally ingested, paraquat is a poison capable of causing a considerable toxicity to lungs, kidney, liver and brain (Smith & Heath, 1976, Fairshter, Rosen & Smith, 1976). It was found that paraquat has a considerable influence on various tissues (Smith, 1985). The lung specificity of paraquat toxic effect is due to the active accumulation of the herbicide in alveolar type I and type II epithelial cells (Smith, 1985). Bus *et al.* (Bus, Aust & Gibson, 1974, 1975) suggested that paraquat may cause damage to the lung alveoli by catalysing the peroxidation of lung lipids.

It is believed that the toxicity of this herbicide is mainly due to the toxic oxygen species formed in the reaction of bipiridylium cation radicals with oxygen (Bus *et al.* 1975; Bus, Cagan, Olgord & Gibson, 1976). Furthermore, the authors suggested that the one electron reduction of paraquat mediated by NADH cytochrome c reductase results in a formation of reduced oxygen species. However, the detailed biochemical mechanism of paraquat toxicity remains unclear. One of the possible explanations of paraquat toxicity involves the assumption that superoxide anion radicals can cause lipid peroxidation which initiates the process of cell membrane damage. In agreement with this view, paraquat is known to stimulate lipid peroxidation in lung microsomes *in vitro* (Bus *et al.* 1974; Trush, Minnaugh, Ginsburg & Gram, 1981).

On the other hand, numerous reports questioned this hypothesis. Shu *et al.* (Shu, Talcot, Rice & Wei, 1979) did not find elevated conjugated dienes in mice lung after paraquat treatment. Kornbust and Mavis (1980) also reported that paraquat did not cause pulmonary toxicity by initiating peroxidation of lung lipids. They suggested that the *in vitro* effect of bipyridinum herbicides may not be related to the mechanisms of their toxicity *in vivo*. Another explanation may concern changes in fatty acid composition, which are likely to disturb membrane lipid structure (Kornbust & Mavis, 1980, Beretta, Fadini, Montesiassa, Pomapa & Stoppani, 1981).

Paraquat is also known to affect antioxidant enzyme activities and induce decrease in the cell glutathione levels (Sandy, Moldens, Ross & Smith, 1986). In this paper the effect of paraquat on human red blood cells was also studied using spin labelling.

MATERIALS AND METHODS

Chemicals

Glutathione, thiobarbituric acid and ascorbic acid were from Sigma Chemical Co. All other chemicals were of analytical grade from POCh

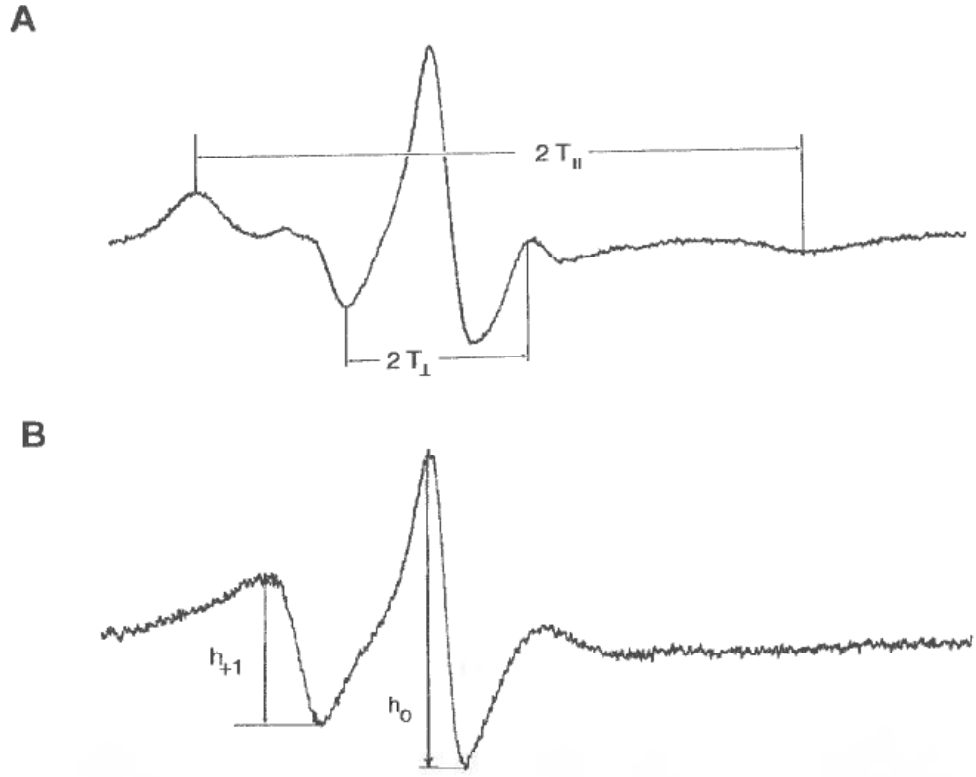


Fig. 1 EPR spectra of methyl 5-doxylpalmitate (a) and methyl 12-doxylstearate (b) incorporated into human reds blood cells. The values of the order parameter (S) and the h_{+1}/h_0 ratio were calculated for both Met 5 DP and for met 12-DS. $T_{\parallel}$ and $T_{\perp}$ are hyperfine splitting constants, h_0 and h_{+1} are midline height and high-field line height, respectively.

(Gliwice, Poland). Paraquat was used as Gramoxone (25% water solution of paraquat).

Methyl 5-doxylpalmitate (Met 5-DP) and methyl 12-doxylstearate (Met 12-DS), were synthesized according to Hubbell and McConnell (1971) and Waggoner *et al.* (Waggoner, Kingzett, Rottschaefer, Griffith & Keith, 1969), respectively. Maleimide spin label (MSL, 4-maleimido-2,2,6,6-tetramethylpiperidine-1-oxyl) was prepared according to Misharin and Polianovskij (1974) and Gaffney (1976).

Erythrocyte and erythrocyte membrane isolation

Human blood was collected from five healthy donors, attending the outpatient clinic of the local hospital.

Erythrocytes were centrifuged and subsequently washed three times with phosphate buffer saline (PBS) pH 7.4. Erythrocyte membranes were prepared at 4°C by hypotonic lysis using 20 mmol/l sodium phosphate buffer pH 7.4. The membrane ghosts were successively washed with 20 mmol/L, 10 mmol/L and 5 mmol/L phosphate buffer, pH 7.4.

Determination of lipid peroxidation

Given the treatment of red blood cells with various concentrations of paraquat, the samples were subjected to analysis of thiobarbituric reactive substances (TBARS) according to the method described by Placer *et al.* (Placer, Cushman & Johnson, 1966) with modifications. The reaction products (TBARS) were extracted with butanol and the absorbance was measured at 532 nm.

Spin labeling of erythrocytes and erythrocyte membranes

Erythrocytes were labeled by the introduction of aliquots of the ethanol solution of spin label fatty acid esters. The final ethanol concentration in erythrocyte suspension was less than 0.05% (v/v). In the case of MSL, erythrocytes were incubated for 1 hr at room temperature, and then the unbound spin label was removed by several washings until the ESR signal in supernatant disappeared.

After labeling, erythrocyte suspensions were incubated at room temperature for 1 hr with increasing concentrations (0.05, 0.1 and 0.3 mmol/L) of paraquat.

ESR measurements were performed at room temperature using SE/X-20 (X-band) spectrometer (Wroclaw Technical University, Poland).

Statistical analysis

Statistical analyses included the calculation of means ± SD. The significance of differences was estimated by Tuckey's test for multiple comparisons.

RESULTS

Fig. 1 a shows a typical spectrum of methyl 5-doxylpalmitate incorporated into human red blood cells. The order parameter, which reflects the membrane lipid fluidity was calculated from the equation (Hubbell & McConnell, 1971):

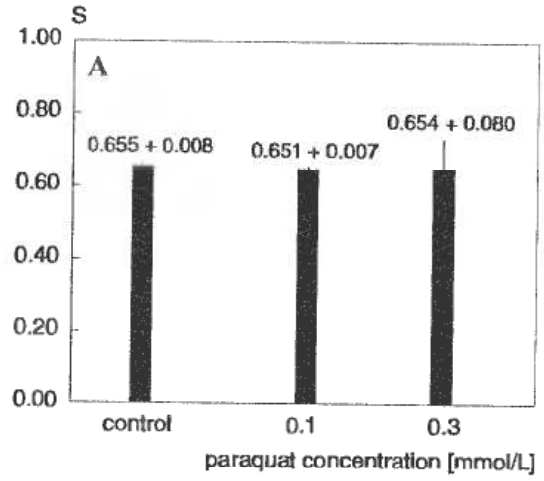
$$S = (T_{\parallel} - T_{\perp})\, a_N / (T_{ZZ} - T_{XX})\, a'_N$$

where $T_{\parallel}$ and $T_{\perp}$ are hyperfine splittings constants for the magnetic field parallel and perpendicular to the bilayer normal, respectively, while $T_{ZZ} = 32.4$ G and $T_{XX} = 6.1$ G are hyperfine splitting parameters determined after the incorporation of nitroxide derivatives into host crystals (Seelig, 1970).

$a'_N = (T_{\parallel} + 2T_{\perp})/3$ and $a_N = (T_{ZZ} + 2T_{XX})$ are the isotropic hyperfine constants for nitroxide in membrane and crystal state, respectively.

We also used methyl 12-doxylstearate which monitors the changes in deeper region of lipid membrane (Fig 1b). The experimental parameter h_{+1}/h_0, where h_{+1} and h_0 are the heights of low-field line and middle line of the spectra, respectively, was determined as a semiquantitative measure of acyl chain flexibility corresponding to lipid bilayer fluidity (Morrisett, Pownall, Plumlee, Smith, Zahner, Esfahani & Wakil, 1975; Stulne-Sekalec & Stanacev, 1978). Figures 2a and 2b show the effects of the increasing concentrations of paraquat on lipid bilayer dynamics. These results indicate that paraquat does not change lipid bilayer near the surface of membrane (as indicated by Met 5-DP). However, paraquat induced a significant increase of the h_{+1}/h_0 parameter for Met 12-DS. This increase is significant (approx. 5%) for methyl 12-doxylstearate, in which the nitroxide reporting group is located deeper, and these changes are relevant to the increase of membrane lipid fluidity.

Fig. 3a shows the spectrum of maleimide spin label attached to human red blood cells. Under neutral pH maleimide reacts mainly with thiol groups of proteins (Berliner, 1983). MSL penetrates membrane and reacts faster with -SH groups



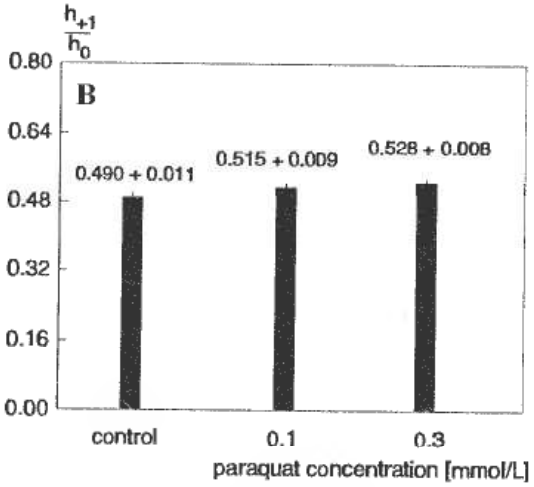


Fig. 2 Effect of different paraquat concentrations on membrane lipid fluidity monitored by Met 5-DP (a) (no significant changes) and Met 12-DS (b) - 0.1 mmol/L and 0.3 mmol/L, $p < 0.001$.

of internal peptides and/or proteins than with membrane proteins (Gwoździński, 1991).

We also showed that more than 90% of label was bound to intracellular fluids.

For a measure of mobility of internal peptides or/and proteins the relative correlation times were calculated from the equation (Keith *et al.*, 1970):

$$\tau_C = k \cdot W_0 \cdot [(h_0 / h_{-1})^{\frac{1}{2}} - 1],$$

where τ_C is a rotational correlation time, k is the constant equal to $6.5 \cdot 10^{10}$ for nitroxide spin label, W_0, h_0 and h_{-1} are mid field line width, mid line height and high field line height respectively. Maleimide spin label attached to intact human erythrocytes yields a triplet spectrum, whereas the spectrum of the label bound to red blood cell

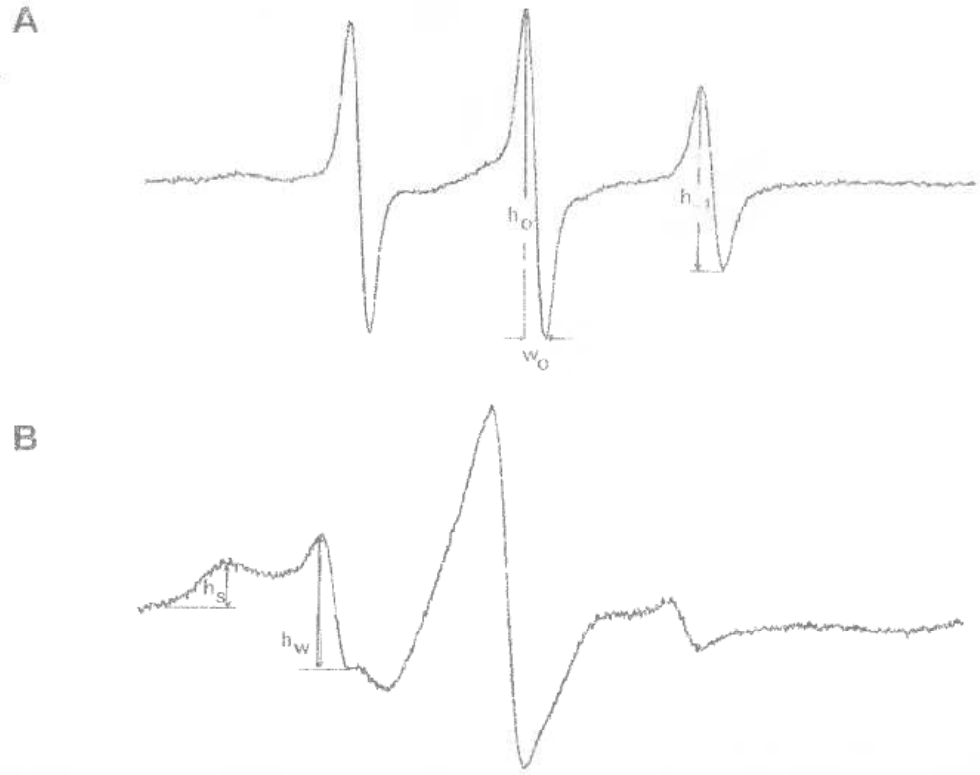


Fig. 3. EPR spectra of 3-maleimido-2,2,6,6-tetramethylpiperidine-1-oxyl attached to human red blood cells (a) and red blood cell membranes (b). The relative correlation time, τ_c, and h_w/h_s ratio were calculated to evaluate the spin labeled proteins mobility. h_w and h_s are weakly-immobilized (narrow-line) and strongly immobilized (broad-line) components, respectively.

membrane reveals the ESR signals of weakly immobilized (narrow-line, hw) and strongly immobilized (broad-line, hs) components (Fig 3b). The analyses of the spectra of maleimide attached to red blood cells membrane was performed by the calculation of h_w/h_s ratio, which is a very sensitive measure of the physical state of proteins in the membrane (Butterfield, 1982; Fung 1983).

Small perturbations in the nearest proximity of the label bound to membrane proteins may produce significant changes in the MSL spectra used for the monitoring of conformational changes of proteins.

Figures 4a and 4b show the effects of various concentrations of paraquat on internal protein mobility and membrane protein mobility, respectively. Using maleimide spin label we observed the increase in h_w/h_s ratio, which reflects the paraquat-induced changes in protein conformation or possibly disposition of spin labeled fragments of proteins. We found the increase in mobility of the label only in the isolated membrane ghosts. The results presented herein suggest the occurrence of possible changes in protein conformation in membranes but not in the intracellular matrix.

We also checked the effect of various substances on paraquat reduction. We found that paraquat can be reduced to paraquat cation radicals by various substances such as ascorbate, glutathione and other thiols. It is possible than these substances may reduce paraquat to its radical and thus the initiate the generation of toxic oxygen species.

We observed the increase of lipid fluidity but we did not find lipid peroxidation in erythrocytes, as estimated by the determination of an amount of TBA-reacting substances, including malondialdehyde.

DISCUSSION

It is generally accepted that paraquat can be reduced to paraquat cation radical:

$$PQ^{++} \rightarrow PQ^{+}$$

Paraquat cation radical is rapidly reoxidized by molecular oxygen with the formation of superoxide anion radical:

$$PQ^{+\cdot} + O_2 \rightarrow PQ^{++} + O_2^{\cdot -}$$

Enzymatic dismutation of superoxide anion radical provides hydrogen peroxide, which in the

Haber-Weiss reaction can generate hydroxyl radical

$$O_2^- + H_2O_2 \rightarrow OH^{\cdot} + OH^- + H_2O_2$$

This reaction, however, is possible only in the presence of traces of heavy metal ions. Furthermore, even more likely is the reaction of paraquat cation radical with hydrogen peroxide:

$$PQ^{+} + H_2O_2 \rightarrow PQ^{++} + OH^{\cdot} + OH^-$$

Depending on the conditions, all the toxic oxygen species mentioned, including singlet oxygen, are likely to appear.

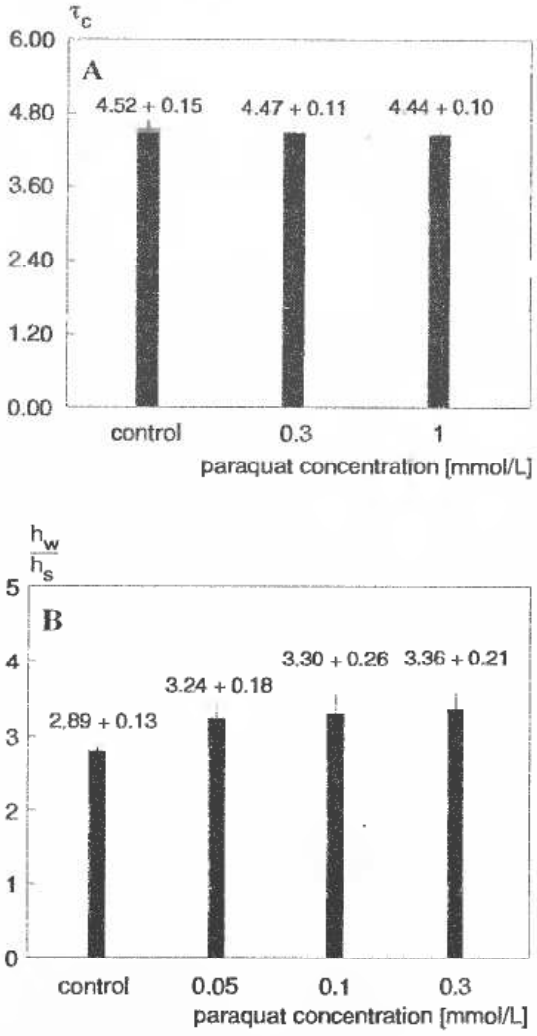


Fig. 4. Paraquat-induced changes in the mobility of maleimide spin labels attached to red blood cells (a) (no significant changes) and red blood cell membranes (b). 0.05 mmol/L, 0.1 mmol/L and 0.3 mmol/L, $p < 0.001$

However, the mechanism of cell injury as induced by paraquat may differ in various organs and depend on whether the study was performed under either *in vivo* or *in vitro* conditions. Paraquat was reported to stimulate lipid peroxidation in microsomes isolated from the murine lungs (Bus *et al.*, 1975) and rats (Trush *et al.*, 1981). In contrast, Shu *et al.* (1979) did not find elevated levels of conjugated dienes (the indicator of lipid peroxidation after paraquat treatment *in vivo* and *in vitro* (Kornbrust & Mavis, 1980) in the lung. Sato *et al.* (Sato, Fijii, Yuge & Morio, 1992) showed increased lipid peroxide levels only in murine liver, but not in lungs, kidney or brain upon the treatment with paraquat.

In this paper we attempted to answer the question whether paraquat-induced lipid peroxidation is correlated with the alterations of membrane fluidity. We showed that paraquat increased lipid fluidity as indicated by methyl 12-doxylstearate spectra in deeper regions of lipid layer. These results correspond very well with our previous results (Gwoździński, 1991b). We observed an increase in membrane lipid fluidity without the concurrent lipid peroxidation after gamma radiation of red blood cells. In contrast, an increased ridigity in all the regions of the bilayer upon lipid peroxidation was shown by others (Bruch & Thayer, 1982; Bartosz, Christ, Bosse, Stephan & Gartner, 1987).

The effect of paraquat on the red cell proteins, using maleimide spin label, was also examined. Paraquat did not cause significant changes in internal peptides or/and proteins. On the other hand, the increase in the h_w/h_s ratio correlated well with the increasing paraquat concentrations in isolated red blood cell ghosts. This increase may reflect the conformational changes of proteins after paraquat treatment.

Our results suggest that the increase in membrane fluidity is not associated with the peroxidation of membrane lipids in red blood cells. It seems that changes in lipid fluidity may be caused by a different mechanism than lipid peroxidation. Such an observation corresponds very well with the previous reports, where considerable changes in the fatty acid composition probably resulted in the weakening of the structure in membrane lipids (Kornbrust & Mavis, 1980; Beretta *et al.*, 1981). We also found the significant alterations in membrane proteins. It is thus likely that paraquat may change protein conformation and the subsequent increase in fluidity may possibly result from the alterations in lipid- protein interaction.

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Current Topics in Biophysics, vol. 18(2), 132-134

THE INFLUENCE OF MICROWAVE POWER ON THE TWO-COMPONENT ESR SPECTRA OF NORADRENALINE-MELANIN

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(Accepted 28 December 1994)

The two types of paramagnetic centers: o-semiquinone and nitrogen free radicals, existing in noradrenaline-melanin were investigated by electron spin resonance method using numerical analysis of lineshape of the ESR spectra. The changes of the ESR linewidths and intensities caused by an increase of microwave power were measured. The homogeneous broadening of the ESR lines was detected. The long and short spin-lattice relaxation time is characteristic for o-semiquinone and nitrogen free radicals, respectively. The interaction of unpaired electrons with lattice atoms is attenuated by presence of atmospheric oxygen in sample environment.

INTRODUCTION

Electron spin resonance studies with continuous wave indicate long spin-lattice relaxation times (10–100 ms) of unpaired electrons in natural and synthetic melanins (Blois, Zahlan & Maling, 1964). Chemical and physical treatments of melanin influence the interactions of unpaired electrons with lattice atoms in the polymers, changing their saturation behaviour. The spin lattice- relaxation time was found to be shortened by copper-doping, because of a cross relaxation effect due to the interaction between copper and the melanin spin system (Blois *et al.*, 1964). Effects on relaxation time due to hydration of the samples was observed (Blois *et al.*, 1964). Homogeneously broadened ESR signal of L-DOPA melanin in solution, in the absence of oxygen, becomes inhomogeneously broadened as a concequence of the magnetic interaction with oxygen (Pascutti, 1992).

The aim of our ESR studies, using progressive microwave power saturation, was to compare spin-lattice interactions of o-semiquinone and nitrogen free radicals in noradrenaline-melanin and determine the influence of atmospheric oxygen on the relaxation process in the investigated melanin.

METHODS

Preparation of the noradrenaline-melanin sample

The investigated model neuromelanin was obtained by oxidative polymerisation of noradrenaline in Tris-HCl buffer (0.05 M, pH 7.4) by method reported previously, Stępień et al. (1989). The solution of the catecholamine sample (5 mM) was aerated for 48 h. After acidification with hydrochloric acid to a final pH of 2, the melanin was separated by centrifugation, dialyzed against distilled water and dried.

ESR measurements

The electron spin resonance investigations were performed using an X-band (9.3 GHz) ESR spectrometer, with modulation of magnetic field of 100 kHz, made by RADIOPAN-Poznań. The microwave frequency was recorded.

The ESR spectra of dry noradrenaline-melanin sample in contact with air were measured. The sample was then evacuated at 10 Torr for 24 h, sealed under vacuum and the ESR spectra were measured again. In order to observe the influence of microwave power on ESR linewidth and intensity measurements were taken with a wide range of attenuation of microwave power (20-0 dB). Numerical analyses of ESR spectra was made using algorithm given by Opfermann (1984).

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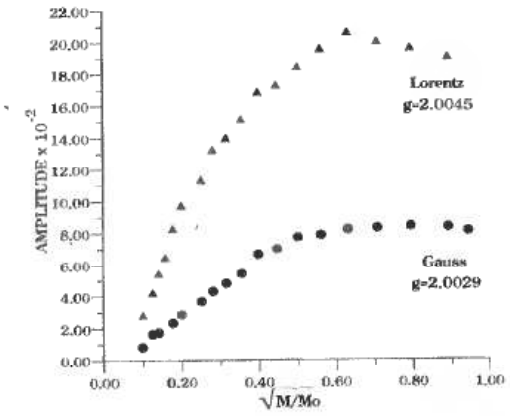

Fig. 1. The influence of microwave power on intensities (in arbitrary units) of ESR signals from o-semiquinone (σ) and nitrogen (λ) free radicals of noradrenaline-melanin, for sample in air. Mo, M - produced by source and used microwave power, respectively (the same remarks in fig. 2-4).

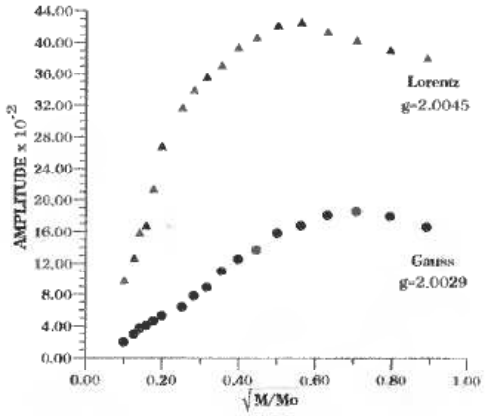

Fig 2. The influence of microwave power on intensities of ESR signals from o-semiquinone (σ) and nitrogen (λ) free radicals of evacuated noradrenaline-melanin sample.

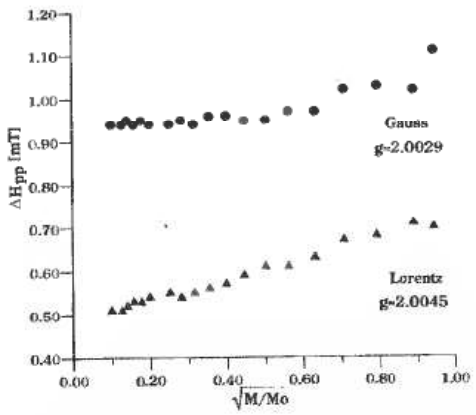

Fig.3. The broadening of the ESR lines from oxygen (σ) and nitrogen (λ) free radicals of noradrenaline-melanin, with increase of microwave power (sample in air).

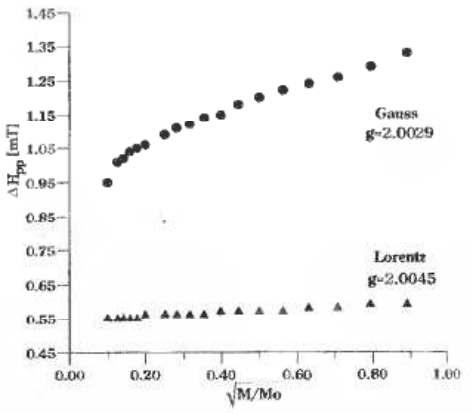

Fig.4. The broadening of the ESR lines from oxygen (σ) and nitrogen (λ) free radicals of noradrenaline-melanin, with increase of microwave power (evacuated sample).

RESULTS AND DISCUSSION

Two types of paramagnetic centers exist in the investigated noradrenaline-melanin: o-semiquinone free radicals with g-value of 2.0045, and probably nitrogen free radicals with lower g-value of 2.0029. The ESR lines of o-semiquinone and nitrogen free radicals are described by Lorentz and Gauss function, respectively. The experimental spectra of noradrenaline-melanin, both for measurements in air and in vacuum, are fitted by superposition of these two curves. The ESR lines of nitrogen free radicals were found to be high broadened by strong dipol interactions (the linewidths of 0.94 mT for sample in air, 20 dB-attenuation of microwave power). The narrower ESR lines (0.51 mT) were measured under the same conditions for oxygen free radicals. Oxygen and nitrogen free radicals interact with one unpaired electron of atmospheric oxygen molecule by weak quasi-chemical bonds.

Homogeneous broadening of the ESR lines of oxygen and nitrogen free radicals was monitored. The intensity of all the ESR signals increases initially with an increase of microwave power, and decreases after saturation (Fig. 1, 2). The ESR lines broaden with increase of microwave power used (Fig. 3, 4).

Unpaired electrons localized on oxygen and nitrogen atoms in noradrenaline-melanin visibly differ in saturation behaviour. The ESR lines of o-semiquinone free radicals with relatively longer

spin-lattice relaxation time saturate for lower values of microwave power than the ESR signals of nitrogen free radicals (Fig. 1, 2). It leads to the conclusion that weak and strong interactions with lattice atoms of noradrenaline-melanin are characteristic for oxygen and nitrogen free radicals, respectively.

The quasi-chemical bonds between unpaired electrons of the two groups in the melanin and atmospheric oxygen change the spin-lattice interactions in the sample. The ESR lines in air saturate for the lower microwave powers (Fig. 1, 2). The spin-lattice relaxation time of unpaired electrons is longer in sample in air than for evacuated sample. The quasi-chemical bonds attenuate interactions of both type of unpaired electrons with the lattice atoms.

Summing up, two group of paramagnetic centers with different magnetic interactions exist in the investigated noradrenaline-melanin. Weak and strong, spin-spin as well as spin-lattice interactions, were found for o-semiquinone and nitrogen free radicals, respectively. Spin-lattice relaxation in the studied melanin is attenuated by atmospheric oxygen.

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Current Topics in Biophysics, vol. 18(2), 135-138

ANTIOXIDANT ACTIVITY OF MODEL NEUROMELANINS IN THE PROCESS OF LIPID PEROXIDATION

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The effect of model neuromelanins on peroxidation of unoxidized and partly oxidized lecithin in liposomal membranes was studied. It was found that dopamine- and noradrenaline – melanins added to unoxidized liposomes effectively suppressed the yield of thiobarbituric acid reactive substances during UV- or Fe^{2+} -induced lecithin peroxidation. When melanin was added to partly oxidized liposomes, prepared by UV irradiation or by preincubation of liposomes with Fe^{2+}, a significant decrease in conjugated diene hydroperoxides formation and TBA-reactive substances accumulation was observed. The obtained results suggest that model neuromelanins are able to inhibit lipid peroxidation, acting as chain - breaking antioxidants.

INTRODUCTION

Neuromelanins, the dark pigment of human brain, are products of the metabolism of dopamine or noradrenaline via the indole pathway (Barden, 1969; Rodgers & Curzon, 1975; Graham, 1978). They accumulate during life in the cytoplasm of the catecholaminergic neurons of the brain-stem and basal ganglia (Mann & Yates, 1974). Their biological functions are still controversial. The vulnerability of melanized catecholaminergic neurons to depigmentation and degeneration in Parkinson's disease (Hirsch, Graybiel & Agid, 1988), and an increase in total nigral content (Sofic, Paulus, Jellinger, Riederer & Youdim, 1991; Dexter, Wells, Agid, Agid, Lees, Jenner & Marsden, 1987) and in basal lipid peroxidation in the substantia nigra of Parkinsonian brains (Dexter, Carter, Agid, Agid, Lees, Jenner & Marsden, 1986, Dexter, Carter, Wells, Javoy-Agid, Agid, Lees, Jenner & Marsden 1989; Jenner, Dexter, Sian, Schapira & Marsden, 1992) suggest that neuromelanins may play an important role in the process of lipid peroxidation in biological membranes. Ben-Shachar *et al.* (Ben-Shachar, Riederer & Youdim, 1991) reported that synthetic dopamine-melanin decreased basal lipid peroxidation in rat cerebral cortex homogenates, but it potentiated lipid peroxidation after addition of iron to homogenates. In contrast, Porębska-Budny *et al.* (Porębska-Budny, Sakina, Stępień, Donstov & Wilczok, 1992) found that melanins obtained from catecholamines were able to inhibit Fe^{2+}-ascorbic acid – induced peroxidation of cardiolipin liposomes. The ability of melanins to suppress lecithin photooxidation was also demonstrated (Stępień, Porębska-Budny, Hollek & Wilczok, 1992).

The aim of the present study was to determine and compare the effect of model neuromelanins on peroxidation unoxidized and partly oxidized lecithin in liposome membranes.

MATERIALS AND METHODS

Model neuromelanins were obtained by oxidative polymerization of dopamine and noradrenaline, as described previously (Stępień, Dworzański, Bilińska, Porębska-Budny, Hollek & Wilczok, 1989). Multilamellar liposomes were freshly prepared from egg yolk lecithin (L-α-phosphatidylcholine, Sigma, type XVI-E) according to Bangham *et al.* (Bangham, Hill & Miller, 1974). The amount of conjugated dienes in the obtained liposomes was negligible as judged basing on absorption values at 232 nm. Lecithin peroxidation was induced by Fe^{2+} (150 mM or 300 mM) or by UV light. (UV lamp POLAMP typ PLK-85, UVA 44%, UVB 33%, UVC 23%, λ_{max} 365 nm, 125 W). During irradiation (30 min), liposome suspensions were continuously stirred and cooled in an ice-water bath. Melanin suspensions in Tris-HCl buffer

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bath. Melanin suspensions in Tris-HCl buffer were added to unoxidized or partly oxidized liposomes. The oxidized liposomes were prepared by UV irradiation of liposomal suspensions or by preincubation of liposomes with Fe^{2+} (300 μM). Final concentration of melanins and lecithin in incubation mixtures were 400 μg/ml and 3.5 mg/ml, respectively.

The accumulation of lecithin peroxidation products was assayed by the thiobarbituric acid (TBA) test as described by Buege and Aust (1978). Since the TBA test is non-specific to malondialdehyde

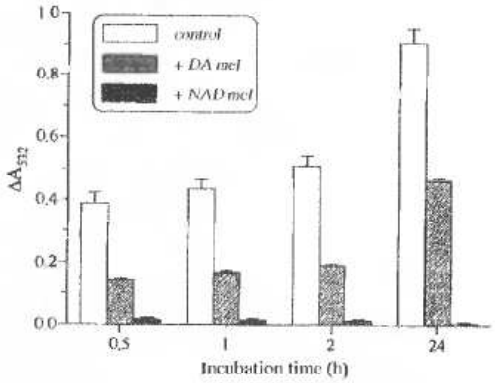

Fig. 1. Effect of dopamine-melanin and noradrenaline-melanin on Fe^{2+}-induced lecithin peroxidation. Fe^{2+} concentration: 150 μM; melanin concentration: 400 μg/ml; lecithin concentration 3.5 mg/ml. Data are mean ± SEM values (n=9).

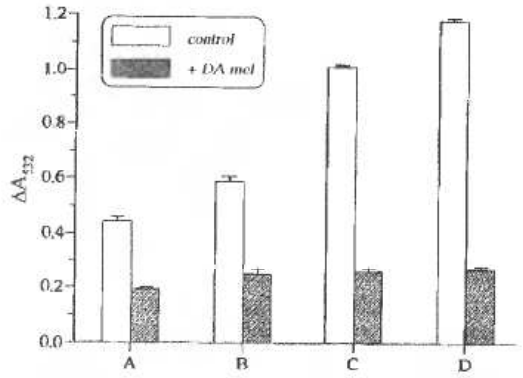

Fig. 2. Effect of dopamine-melanin on peroxidation of oxidized lecithin liposomes prepared by Fe^{2+} addition. After 30 min of preincubation of liposomes with Fe^{2+}, melanin or Tris-HCl buffer were added (A), and liposomes were incubated for 60 min (B), 24 h (C), and 48 h (D). Fe^{2+} concentration: 150 μM; melanin concentration: 3.5 mg/ml. Data are mean ± SEM values (n=9).

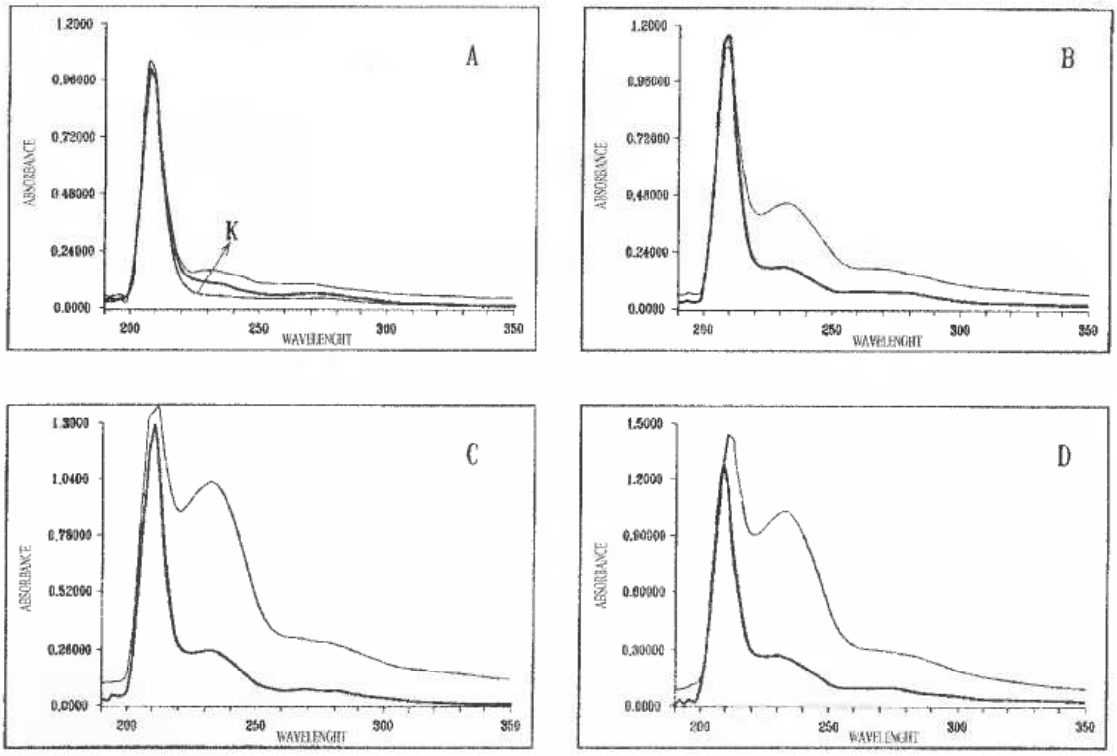

Fig. 3. Ultraviolet spectra of lecithin during peroxidation in the absence (–) and in the presence of dopamine-melanin (-). (A) after preincubation of liposomes with Fe^{2+} and addition of melanin (400 μg/ml); curve K – unoxidized lecithin; (B), (C), (D) after incubation for 24 h, 48 h and 120 h, respectively (see Fig. 2).

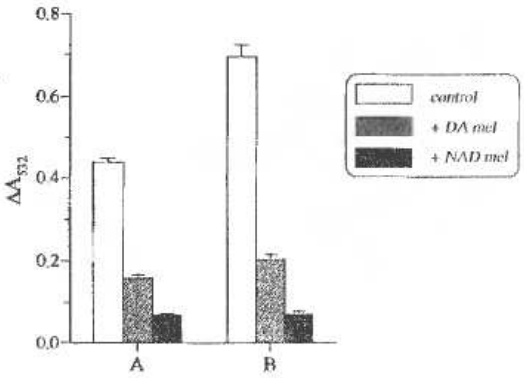

Fig. 4. Effect of dopamine-melanin and noradrenaline-melanin on UV-induced lecithin peroxidation. Suspensions of liposomes (lecithin concentration 3.5 mg/ml) and melanin (400 μg/ml) were irradiated by UV light for 30 min (A) and then incubated for 24 h (B). Data are mean + SEM values (n=9).

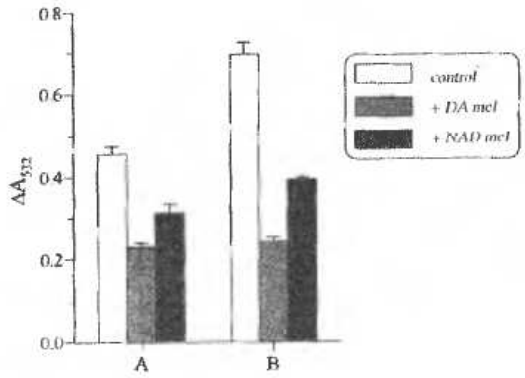

Fig. 5. Effect of melanins on peroxidation of oxidized lecithin liposomes prepared by UV light irradiation for 30 min. Tris-HCl buffer and dopamine- or noradrenaline-melanin (400 μg/ml) were added after irradiation (A) and liposomes (lecithin concentration 3.5 mg/ml) were incubated for 24 h (B). Data are mean + SEM values (n=9).

in peroxidized lipids (Kosugi & Kikugawa, 1989), the degree of lecithin peroxidation was expressed as an increase in absorbance at 532 nm.

The extent of lecithin peroxidation in the absence or presence of melanin was also monitored spectrofotometrically. The 0.2-ml aliquots of incubation mixtures were added to 2.8 ml of absolute ethanol and UV spectra were recorded (Dode-Array HP 8452A spectrophotometer).

RESULTS AND DISCUSSION

The effect of melanins obtained from dopamine and noradrenaline on Fe^{2+}-induced peroxidation of lecithin in liposome membranes is shown in Fig. 1. Melanins were added to unoxidized liposomes before addition of Fe^{2+}. The presence of melanins during incubation of liposomes caused a significant decrease in the yield of thiobarbituric acid – reactive substances compared to the control, indicating their ability to inhibit lipid peroxidation. Antioxidant efficiency of noradrenaline-melanin was higher than dopamine-melanin. The formation of TBA-reactive substances was almost completely suppressed in the presence of noradrenaline-melanin, whereas melanin from dopamine inhibited lecithin peroxidation by about 60%.

When dopamine-melanin was added to partly oxidized lecithin prepared by preincubation of liposomes with Fe^{2+}, a decrease of TBA-reactive substances was observed (Fig. 2A). Since the most of these substances is formed from degradation of various lipid peroxides under the TBA assay conditions, the obtained results suggest that melanin was able to interact with products of lecithin peroxidation and break free radical chain oxidation. Indeed, during subsequent incubation for 48 h the production of TBA-reactive substances was strongly inhibited (Fig. 2,B, C, D).

The course of Fe^{2+}-induced lecithin peroxidation in the absence and presence of dopamine-melanin was also monitored spectrofotometrically. As shown in Fig. 3, oxidation of lecithin in control liposomes was accompanied by an increase in absorbance at 232 nm due to conjugated diene hydroperoxides formation and at 270 nm, arising from conjugated trienes and carbonyl groups of final products of peroxidation. The addition of melanin to partly oxidized liposomes prevented the formation of conjugated dienes and trienes, suggesting that melanin could scavenge lipid alkyl radicals or lipid alkoxyl radicals generated during iron – catalyzed decomposition of lipid hydroperoxides.

The effect of melanins on UV-induced lecithin peroxidation is shown in Fig. 4. When melanin was present durring irradiation of liposomes, a significant inhibition of lecithin photooxidation was found. After 30 min of irradiation, the amount of TBA-reactive substances was reduced to 36% in the presence of dopamine-melanin and to 15% in the presence of noradrenaline-melanin. All the samples were incubated additionally for 24 h at room temperature without irradiation. In the absence of melanin a further increase in lecithin peroxidation was observed, whereas the presence of melanins inhibited the peroxidation. Melanins added to liposomes after UV irradiation also caused a decrease in formation of TBA-reactive substances but in this case the inhibiting effect was lower than that observed when melanin was present from the beginning of irradiation (Fig. 5.).

The obtained results indicate that model neuromelanins are able to inhibit lipid peroxidation, acting as chain-breaking antioxidants. The ability of melanins to bind iron ions (Ben-Shachar *et al.*, 1991), scavenge oxygen free radicals (Sarna, Pilas, Land & Truscott, 1986; Korytowski, Kalyanaraman, Menon, Sarna & Scaly, 1986), and absorb and transform electromagnetic radiation to heat (McGinnes & Proctor, 1973), suggests that melanins may also act as preventive antioxidants.

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Current Topics in Biophysics, vol. 18(2), 139-142

EFFECT OF AMINO ACID PEROXIDES ON THE ERYTHROCYTE MEMBRANE

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(Accepted 28 December 1994)

The effect of amino acid peroxides, reintively stable products of irradiation of amino acid solutions, on erythrocyte membrane was studied. Interaction of proline, lysine, valine and leucine peroxides (100-300 M) with erythrocyte membranes brought about a decrease of membrane protein -SH group content and of activities of (Na^+, K^+)-ATPase and Ca^{2+}-ATPase, and induced aggregation of membrane proteins, due mainly to the formation of interpeptide disulfides. The effects of amino acid peroxides are similar to those of *t*-butyl hydroperoxide. These results indicate that peroxides of amino acid and proteins which can also be formed under physiologic conditions, may be mediators of the cellular action of reactive oxygen species.

INTRODUCTION

Reactions of reactive oxygen species with amino acids and proteins lead to formation of peroxides (Gębicki & Gębicki, 1993). Some amino acids, especially Val, Leu, Ile, Pro and Glu form peroxides with comparatively high yields. In the absence of other reactants, peroxides of proteins and amino acids are relatively stable species but they may react with biological reductants and metal ions (Babiy, Gebicki & Gebicki, 1993; Gebicki & Gebicki, 1993). It is therefore of interest if they may interact with and damage other macromolecules and cellular structures. The aim of this study was to examine the effect of amino acid peroxides on the erythrocyte membrane.

MATERIAL AND METHODS

Amino acid peroxides were generated by irradiation of 20 mM solutions of amino acids (Pro, Val, Leu, Ile, Lys, Glu) prepared in 20 mM phosphate buffer, pH 7.4, phosphate-buffered saline, pH 7.4 (PBS) or deionized water (pH 7.4). Irradiation was performed with a dose of 2.5 kGy of gamma radiation (50 min) from a Co^{60} source while passing oxygen bubbles through the solution during radiolysis. Hydrogen peroxide was removed from irradiated solutions by adding catalase to the final concentration of 2 μg/ml (corresponding to an activity of 60 Sigma units/ml).

Concentration of peroxides formed was determined by the iodometric method (Hicks & Gebicki, 1979; Simpson, Narita, Gieseg, Gebicki, Gebicki & Dean, 1992). Peroxides were reduced with excess of sodium borohydride, excess of borohydride being removed by acid treatment. For comparison, hydrogen peroxide and t-butyl hydroperoxide (both 300 μM) were also used.

Fresh human blood from normal adult donors was obtained in a local blood bank. The erythrocyte were isolated by centrifugation at 4°C, 2 000 × g and purified by three cycles of resuspension and washing with 20 volumes of phosphate-buffered saline (PBS) with 1 mM EDTA and 0.5 mM PMSF. After careful removal of the the buffy coat, the residual leukocytes were removed by passing through a column of HBS cellulose.

For the studies of hemolysis, erythrocytes at the concentration of 0.02% were incubated with amino acid peroxides for up to 48 h at 4°C and hemolysis was determined turbidimetrically at $\lambda = 710$ nm. The erythrocyte ghosts were prepared from washed cells according to Dodge *et al.* (Dodge, Mitchell & Hanahan, 1963) with some modifications. For determination of ATPase activity the ghosts were prepared in Tris-HCl buffer. Formation of protein aggregates and decrease in the content of main protein bands in the erythrocyte ghosts was estimated by SDS-PAGE in the buffer system of Laemmli (1970). Slab gels were prepared in a Desaga electrophoresis unit. The

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Table 1
Peroxide concentration in irradiated amino acid solutions (mean ± SD, number of determinations in parentheses)

Agent	Peroxides [μM]
Buffer	13 ± 5 (3)
Pro	290 ± 31 (4)
Lys	240 ± 57 (5)
Val	220 ± 20 (3)
Leu	166 ± 28 (4)
Glu	90 (1)

gels were stained with Coomassie Brilliant Blue R-250 by the method of Fairbanks *et al.* (Fairbanks, Steck & Wallah, 1971) and analysed with a DESAGA CD-60 densitometer. The major protein bands were numbered 1-7 according to Steck (1974). The total –SH group content of membrane proteins was determined according to Ando and Steiner (1973).

For studies of the effects of peroxides on the erythrocyte membrane enzymatic activities, samples of erythrocyte ghosts (protein concentration of 7.0 mg/ml) were mixed with control solutions and peroxides at a ratio of 1:3. After 0 (control), 8 and 24-hour incubation at room temperature enzymatic activities of the membrane were measured. Na^+, K^+-ATPase activity was determined in a medium containing 100 mM Tris-HCl, pH 7.4, 10 mM Mg^{2+}, 15 mM KCl, 85 mM NaCl and 2 mM ATP (final concentrations), by measurement of the difference in the liberation of inorganic phosphate in the absence and in the presence of

Table 2
Effect of peroxides on the thiol group content of erythrocyte ghosts

Agent	Thiol groups [nmol/mg membrane protein]	P
Control buffer	85.0 ± 1.8 (9)	
Irradiated buffer	83.9 ± 2.0 (9)	NS[a]
H_2O_2	86.5 ± 2.0 (20)	NS[a]
t-But-OOH	65.4 ± 2.0 (18)	<0.0001[a]
Control Pro	81.5 ± 1.8 (20)	
Pro-OOH	68.4 ± 2.3 (20)	<0.0001[b]
Control Leu	74.8 ± 3.8 (10)	
Leu-OOH	65.4 ± 7.0 (10)	<0.002[b]
Control Lys	73.4 ± 0.9 (4)	
Lys-OOH	67.8 ± 2.9 (4)	0.05[b]

[a] With respect to control buffer
[b] With respect to non-irradiated amino acid

0.1 mM ouabain during 30-min incubation of a membrane preparations at 37°C. Ca^{2+}-ATPase activity was measured in a medium containing 100 mM Tris-HCl, pH 7.4, 10 mM Mg^{2+}, 1 mM EGTA, as a difference in the liberation of inorganic phosphate in the presence and in the absence of 1 mM Ca^{2+}. The phosphate liberated was estimated with Malachite Green (Baykov, Evtushenko & Avaeva, 1988). Acetylcholinesterase activity was determined according to Ellman *et al.* (Ellman, Courtney, Andres & Featherstone, 1961).

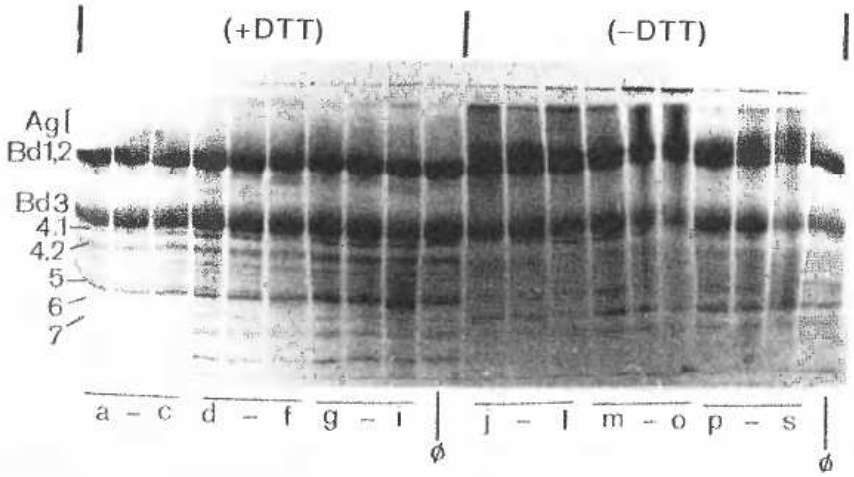

Fig. 1. SDS-PAGE of proteins of control erythrocyte membranes and of membranes treated with amino acid peroxides for various time periods. Left side: electrophoresis under reducing conditions (in the presence of DTT), right side: electrophoresis under non-reducing conditions; Agg - aggregates. a-c and j-l: Glu-OOH, incubation for 0, 24 and 48 h; d-f and m-o: Lys-OOH, incubation for 0, 24 and 48 h; g-i and p-s: Leu-OOH, incubation for 0, 24 and 48 h.

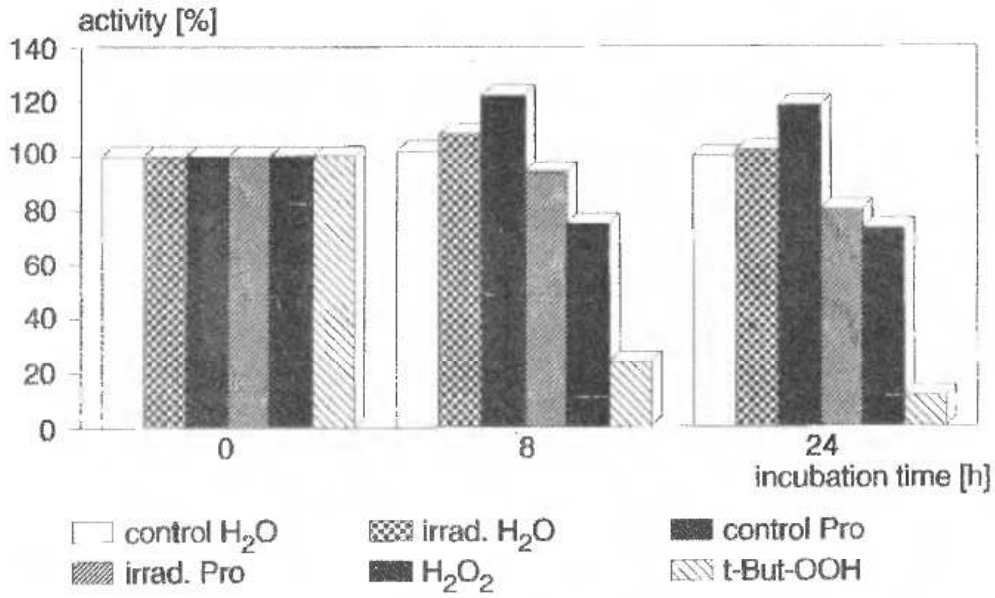

Fig. 2. Effect of peroxides on the Na^+, K^+-ATPase activity of erythrocyte membranes. Control samples include membranes incubated in water, irradiated water and non-irradiated proline solution. Control value of activity: 185 ± 32 nmol/mg protein x h. Mean values of 2 experiments.

RESULTS AND DISCUSSION

Irradiation of solutions of Lys, Pro and Val under the indicated conditions resulted in the formation of peroxides in the range of 200-300 μM while the yield of formation in Leu and Glu solutions was slightly lower (Table 1).

The effects of so formed amino acid peroxides on the following erythrocyte parameters were studied: hemolysis, membrane -SH group content, membrane protein aggregation, membrane protein conformation using a maleimide-TEMPO spin label, activities of membrane ATPases and acetylcholinesterase, and hemoglobin oxidation. No induction of hemolysis by the peroxides (Pro-OOH, Lys-OOH and Glu-OOH) and no effects of the peroxides on acetylcholinesterase activity (Pro-OOH, Lys-OOH), membrane lipids peroxidation and protein conformation as judged from ESR spectra of spin-labeled membranes (Pro-OOH, Lys-OOH) were found (not shown).

SDS-PAGE demonstrated peroxide-induced formation of protein aggregates accompanied by a decrease in the content of Band 1 and 2 (spectrin), Band 3 and Band 4.2. Figure 1 shows the SDS-PAG electrophoregrams of erythrocyte ghosts after incubation at 4°C with peroxides of Glu-OOH, Lys-OOH and Leu-OOH for 0, 24 and 48 hours. The content of protein aggregates was much higher under non-reducing conditions than after reduction with dithiothreitol which demonstrates a predominant contribution of -S-S- bridges to the aggregate formation. The effectiveness of amino acid peroxides for membrane protein aggregate formation was Pro-OOH = Lys-OOH > Leu-OOH > > Val-OOH > Glu-OOH.

Pro-OOH, Leu-OOH and Lys-OOH induced a statistically significant decrease in membrane protein -SH group content (Table 2). The decrease of the membrane -SH group content took place within the initial 60-min incubation of membranes with the peroxides at room temperature.

A significant inhibition of the Na^+, K^+-ATPase activity by the proline hydroperoxide was found (Fig. 2). The Ca^{2+}-ATPase activity decreased gradually also in control preparations, perhaps due to proteolysis which is not completely inhibited in membrane preparations even in the presence of EDTA and PMSF (Bartosz & Gaczyńska, 1985). Nevertheless, the rate of activity decrease was higher in the presence of proline hydroperoxide than in control preparations (membranes added with water) and in membranes added with non-irradiated proline (Fig. 3). Hydrogen peroxide brought about a similar, and t-butyl hydroperoxide a much higher inactivation of the both ATPases.

The effects brought about by amino acid peroxides were similar to those of t-butyl hydroperoxide though lower in spite of similar concentrations. However, being polar and charged, and less membrane-penetrating than the latter, amino acid peroxides may exert a higher degree of selectivity in their reactions and perhaps function as second

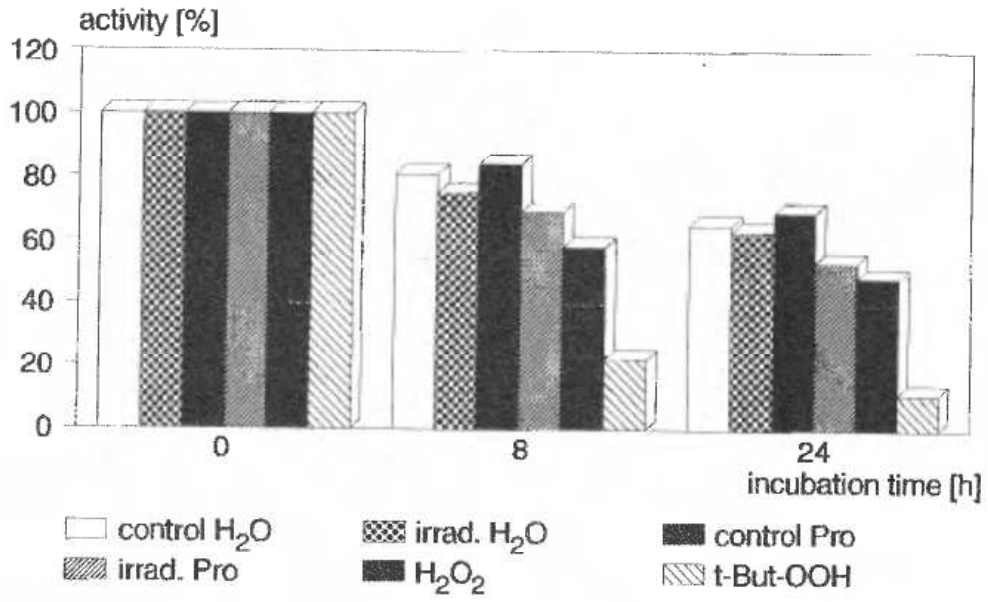

Fig. 3. Effect of peroxides on the Ca^{2+}-ATPase activity of erythrocyte membranes. Control samples include membranes incubated in water, irradiated water and non-irradiated proline solution. Control value of activity: 732 ± 65 nmol/mg protein x h. Mean values of 2 experiments.

messengers of biological damage by reactive oxygen species.

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Current Topics in Biophysics, vol. 18(2), 143-146

RADIATION RESISTANCE OF YEAST MUTANTS LACKING ANTIOXIDANT ENZYMES

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(Accepted 28 December 1994)

Four strains of yeast *Saccharomyces cerevisine* differing from each other in deficiency of antioxidant enzymes such as catalase, copper and zinc superoxide dismutase (ZnCuSOD) or manganese dismutase (MnSOD) and a strin with reduced glutathione level were irradiated by gamma rays. Survival at various doses for each strain was determined by plating suitable cell dilutions on YPG agar plates. Radiation resistance of the strains mentioned above increased as follows: SP4 (wild-type), A50 (catalase-deficient mutant), C4 (glutathione-poor mutant), DSCD1-!C, (ZnCuSOD-deficient mutant), DSCD6-6B (double mutant without either ZnCuSOD or MnSOD). Heat shock induced radiation resistaace was investigated in order to answer the question why the mutant strains lacking some antioxidant enzymes and the strain of reduced glutathione level are significantly less radiosensitive than the wild type. Survival the wild type increased about 4 fold, of catalase-deficient mutant about 3 fold, and of glutathione-poor mutant about 2 fold. Heat shock hardly changed survival of dismutases-deficient mutants. The results suggest that the absence of an antioxidant enzyme or reduced glutathione level may be a form of stress responsible for induction of protective response which markedly increases the radiation resistance of the mutant strains in comparison with the wild type. This phenomenon seems not to depend on either superoxide dismutases or catalase levels because there is no correlation between SOD levels or heat shock induced change of catalase level and survival of various yeast strains before and after heat shock.

INTRODUCTION

Ionizing radiation is one of the means for generating reactive oxygen species (in the air: $G(^{\cdot}OH)=2.7$; $G(O_2^{\cdot-})=3.3$; $G(H_2O_2)=0.71$) which are able to kill cells, causing oxidation of proteins (Davies, 1986), DNA lesions (Herskind & Westergaard, 1986; Ewing, Koval & Walton, 1986; Radford, 1986) and lipid peroxidation (Dix & Aikens, 1993).

Catalase, superoxide dismutases (both ZnCuSOD or MnSOD) and glutathione are known to be important as the antioxidant agents (Krinsky, 1992). In yeast protection against oxidant attack is mainly afforded by superoxide dismutases (Biliński, Krawiec, Liczmański & Litwińska, 1985) while the protective role of catalases is not clear (Biliński, Krawiec, Litwińska & Blaszczyńska, 1988). SOD-deficient mutants are highly sensitive to oxygen stress induced by 100% oxygen, paraquat and illumination of riboflavin but less sensitive to hydrogen peroxide. In contrast, catalaseless mutants do not differ in oxysensitivity from the wild-type.

In this paper four strains of yeast *Saccharomyces cerevisiae* differing from each other in the amount of antioxidant enzymes, and a glutathione-poor strain were investigated in order to determine if lack of catalase, ZnCuSOD, MnSOD or reduced level of a glutathione have an influence on radiation survival.

MATERIAL AND METHODS

Strains

The organism used in these experiments was the yeast Saccharomyces cerevisiae. Wild type and mutants were obtained from Zamość College of Agriculture: SP4 (wild-type), A50 (catalase-deficient mutant), C4 (glutathione-poor mutant), DSCD1-1C (ZnCuSOD-deficient mutant), DSCD6-6B (double mutant without either ZnCuSOD or MnSOD).

Radiation exposure

The yeast strains after having grown in liquid medium YPG (1% yeast extract, 1% peptone and 2% glucose) at 22°C to mid log phase (1×10^7

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This study was supported by the Committee for Scientific Research (Grant No.6 P203 001 04).

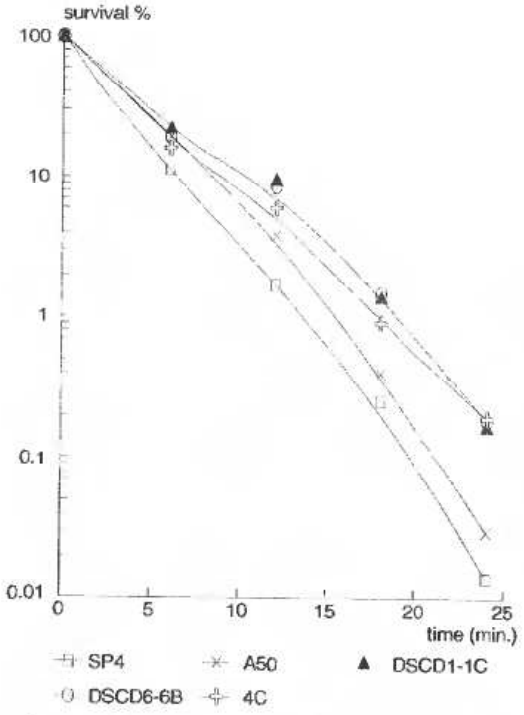

Fig. 1 Survival curves of gamma-irradiated *Saccharomyces cerevisiae*

cells/ml) were irradiated (25 ml in a 250-ml flask) at ambient temperature by gamma rays for 6, 12, 18, 24 minutes at a dose rate of 0.925 Gy/s. Survival at various doses for each strain was determined by plating suitable cell dilutions on YPG agar plates (1% yeast extract, 1% peptone, 2% glucose, 2% agar).

Heat shock-induction of radiation tolerance

The yeast strains were heat shocked by transfer of the culture (25 ml in a 250-ml flask) to a shaking water bath at 37°C for 30 min. Survival of each strain was determined at the dose of 666 Gy before and after heat shock.

Cell-free extracts

Cell-free extracts were obtained by shaking of

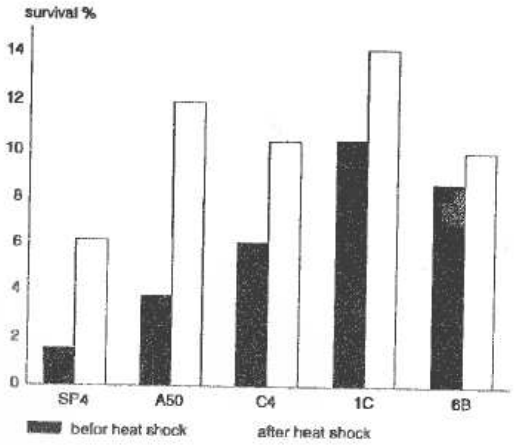

Fig. 2 Survival of *Saccharomyces cerevisiae* irradiated 666 Gy before and after heat shock

yeast paste with glass beads and centrifugation at 3500 rpm during 25 min.

Catalase activity

Catalase activity was determined by the method of Beers and Sizer (1952) in which catalase activity is defined in terms of nanomoles of hydrogen peroxide consumed per minute per milligram of protein sample (change in absorbance at 240 nm/min).

SOD activity

SOD activity was determined by the method of Oberley and Spitz (1985) in which one unit of SOD activity is the amount of protein which gives 50% inhibition of NBT reduction by $O_2^{\cdot}$ (measured by change in absorbance at 540 nm/min).

Enzyme activities are expressed as units per milligram of protein, with protein measured by the method of Lowry (1951).

Catalase and SOD activities were measured for each strain before and after heat shock either for irradiated (666 Gy) or for not-irradiated samples. Each experiment was repeated at least three times from independent cultures.

Table 1. The per cent survival at the dose of 666 Gy.

Strain		Survival
SP4	wild-type	1.7% ± 0.4%
A50	catalaseless	3.8% ± 1.0%
C4	glutathione-poor	6.0% ± 0.9%
DSCD1-1C	ZnCuSOD-less	9.4% ± 1.8%
DSCD6-6B	ZnCuSOD, MnSOD-less	8.5% ± 1.3%

Table 2. Catalase activity [U/mg of protein]

Strain:	SP4	A50	4C	DSCD1-1C	DSCD6-6B
–	0.36 ± 0.03	0	2.34 ± 0.13	0.20 ± 0.05	0.22 ± 0.06
666 Gy	0.34 ± 0.06	0	2.79 ± 0.06	0.28 ± 0.04	0.25 ± 0.04
37°C, 30 min	3.29 ± 0.10	0	5.45 ± 0.13	2.70 ± 0.11	2.60 ± 0.17
37°C, 30 min, 666 Gy	2.83 ± 0.17	0	5.50 ± 0.08	2.60 ± 0.03	2.60 ± 0.09

Table 3. SOD activity [U/mg of protein]

Strain:	SP4	A50	4C	DSCD1-1C	DSCD6-6B
–	84.7 ± 9.1	63.1 ± 12.5	61.5 ± 7.6	42.3 ± 1.3	32.0 ± 1.2
666 Gy	84.3 ± 6.0	49.9 ± 2.6	65.1 ± 11.7	47.6 ± 3.6	34.6 ± 3.4
37°C, 30 min	122.1 ± 4.1	78.9 ± 8.7	112.5 ± 6.3	49.7 ± 5.5	35.2 ± 9.1
37°C, 30 min, 666 Gy	92.4 ± 12.9	76.0 ± 5.8	105.9 ± 16.5	43.1 ± 11.9	46.8 ± 5.7

RESULTS

The mutant strains lacking some antioxidant enzymes and the strain of reduced glutathione level were significantly less sensitive to irradiation than the wild type. The diferences in radiosensitivity among the mutants were smaller and statistically significant at the dose of 666 Gy and 999 Gy. There was no significant difference in survival between SOD-deficient strains (Fig.1, Table 1).

After the heat shock of the wild-type SP4 survival at the dose of 666 Gy increased about 4 fold, that of catalase-deficient mutant A50 about 3 fold, and that of glutathione-poor mutant C4 about 2 fold. Heat shock hardly changed survival of dismutase-deficient mutants: DSCD1-1C, DSCD6-6B (Fig.2).

Enzyme activities of the mutants irradiated after and before heat shock were comparable to those of the wild-type (Tables 2, 3).

DISCUSSION

Mutations leading to deficiency of antioxidant enzymes such as catalase, superoxide dismutases or to reduced level of glutathione have pleiotropic effects including amino acid auxotrophy (Biliński *et al.*, 1985) and radioresistance. The absence of SOD may be involved in hypersensitivity to oxygen (Biliński *et al.*, 1988).

The results of this study suggest that deficiency or reduced level of antioxidants may be a form of stress capable of protective response induction which significantly increases the radiation resistance of the mutant strains in comparison with the wild-type strain. This raises questions of the mechanisms and components of antioxidant shortage-stress response.

The results indicate that there are differences in these mechanisms among the mutants. SOD deficiency result in hypersensitivity to oxygen and radioresistance which might be a consequence of oxygen stress. Physiological studies suggest that the protective response of yeast cells to SOD lack may partly overlap the heat shock response since radioresistance does not change after heat shock.

In contrast, catalases in yeasts seem not to play a protective role against oxygen stress and this would explain the fact that catalase deficiency could not induce defence response to such an extent as SOD deficiency could. Moreover the heat shock does change radioresistance of cataloseless mutants which suggests that catalase lack-defence response differs from that to heat shock and that to SOD lack.

The phenomenon of heat shock-induced radioresistance does not seem to depend on either superoxide dismutases or catalase levels because there is no correlation between SOD levels or heat shock-induced change of catalase level and survival of various irradiated yeast strains before and after heat shock.

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Current Topics in Biophysics, vol 18(2), 147-150

INFLUENCE OF ALCOHOL RADICALS ON THE LEVEL OF DISSOCIATION AND FUNCTIONAL CHARACTERISTICS OF HUMAN HEMOGLOBIN*

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(Accepted 28 December 1994)

In this work the effect of ethanol, 1-butanol and t-butanol radicals generated by radiation on the level of dissociation and on the functional characteristic of human hemoglobin was studied. Dissociation and aggregation of hemoglobin was determined by the method of molecular filtration through Sephadex G-100. Hemoglobin irradiated under anaerobic conditions without alcohol underwent considerable aggregation. The presence of alcohols during irradiation prevented aggregation and a considerable part of protein occured in the dissociated form as dimers. The greatest dissociation was caused by t-butanol radicals under N_2O. In general, all irradiated hemoglobin preparations were characterized by an increased oxygen affinity and decreased co-operativity. The results obtained indicate that alcohol radicals having the odd electron situated on β-carbon cause hemoglobin destruction than those having the odd electron on α-carbon.

INTRODUCTION

In studies of the radiolysis of biomolecules in water solutions alcohols are often used as scavengers of hydroxyl radicals (·OH). Hydroxyl radicals react with alcohols mainly through the abstraction of H atoms causing their transformation into secondary alcohol radicals. Alcohol radicals are less reactive then ·OH radicals but literature data indicate that they are able to damage biomolecules (Schuessler, 1975; Szweda-Lewandowska & Puchala, 1981). Therefore, examination of their destructive influence on biological compounds is important.

Rate constants for the reaction of ·OH with alcohols are: for ethanol $1.8 \cdot 10^9$ $dm^3 mol^{-1} s^{-1}$, for 1-butanol $3.6 \cdot 10^{10}$ dm^3 mol^{-1} s^{-1} and for t-butanol $5.2 \cdot 10^8$ dm^3 mol^{-1} s^{-1} (Dorfman & Adams, 1973). At neutral pH the yield of H-abstraction from the C_α position of ethanol is 83 per cent while the yield of abstraction from the C_β and C_γ carbons only 13%. However, for 1-butanol the yield of H-abstraction from C_α is 53 per cent and for C_β and C_γ together 46 per cent. In the case of t-butanol the abstraction of H in position C_β is 96 percent (Asmus, Mockel & Henglein, 1973).

In this work the effect of alcohol radicals differing in localization of the unpaired electron on the level of dissociation and on the functional characteristics of human hemoglobin was studied. The radicals used were ethanol, 1-butanol and t-butanol radicals generated by radiation.

MATERIAL AND METHODS

Aqueous solutions of human deoxyhemoglobin (2 $mg \cdot cm^{-3}$, i.e. $3.1 \cdot 10^{-5}$ $mol \cdot dm^{-3}$ with respect to tetramer, in 0.02 $mol \cdot dm^{-3}$ phosphate buffer, pH 7) were irradiated at room temperature under the atmosphere of argon or N_2O without alcohols or in the presence of ethanol (0.1 $mol \cdot dm^{-3}$), 1-butanol (0.05 $mol \cdot dm^{-3}$) and t-butanol (0.1 $mol \cdot dm^{-3}$). Under these conditions about 99 per cent of ·OH radicals were scavenged by alcohols. The source of radiation was ^{60}Co and the dose rate was 5.1 $kGy \cdot h^{-1}$.

The level of hemoglobin dissociation and aggregation was studied using the method of molecular filtration through a Sephadex G-100 column (95 × 2.5 cm). Mathematical analysis of the obtained results was carried out using the approximation of peaks by exponentially modified Gauss function (Foley & Dorsey, 1983).

The functional properties of the preparations of hemoglobin were determined on the basis of measurements of oxygen dissociation curves by the spectrophotometric method according to the procedure given in a previous paper (Szweda-Lewandowska & Puchala, 1981).

* This work was supported by State Committee for Scientific Research (Poland), grant No: 4 4540 92 03.

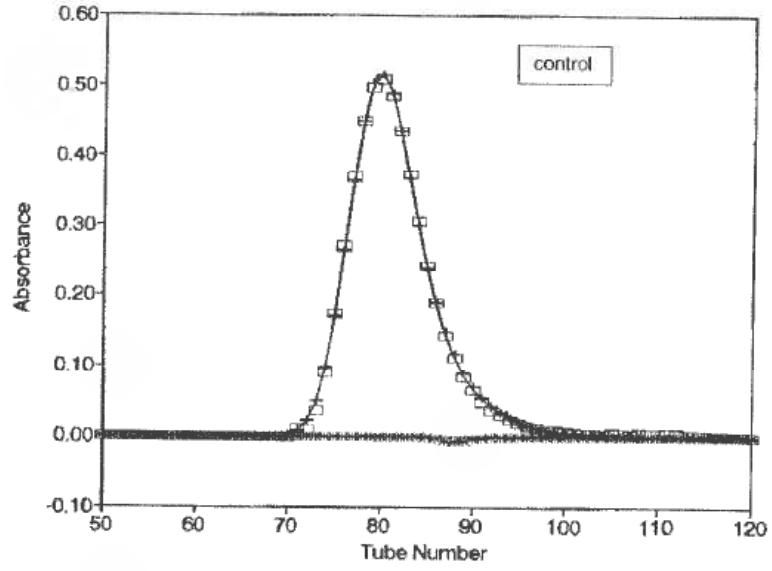
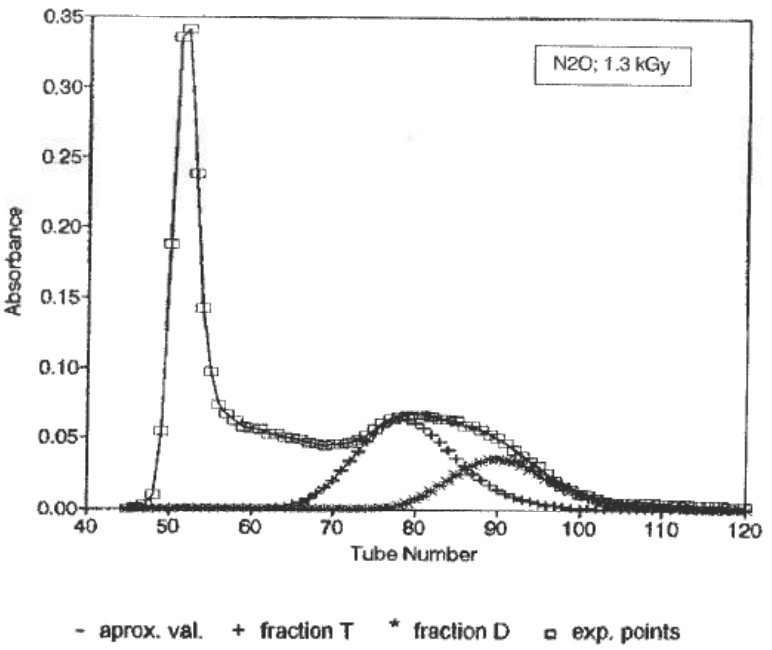


Fig 1. Gel filtration on Sephadex G-100 of control hemoglobin and of hemoglobin irradiated under N_2O with a dose 1.3 kGy.



- aprox. val. + fraction T * fraction D ▫ exp. points

RESULTS AND CONCLUSIONS

Gel filtration

Fig 1. shows elution profiles from a Sephadex G-100 column of hemoglobin solutions irradiated under N_2O with a dose 1.3 kGy, in comparison with unirradiated hemoglobin (control). Control hemoglobin was eluted in the form of one exponentially modified Gauss peak (fraction T) with maximum in tube No 80 which corresponded to tetrameric Hb molecules being dissociated into dimers only to a small extent.

Hemoglobin irradiated under anoxic conditions i.e. under N_2O or argon in the absence of alcohol underwent considerable aggregation which is visible through appearance of the elution peak with maximum in tubes 48–70, with a molecular mass above 120 000 (A). A small part of protein was also eluted in tubes above 87 which indicates the presence of dissociated Hb molecules (dimer fraction D, Fig. 1, Tab. 1).

The presence of alcohols totally prevented aggregation of hemoglobin. Elution profiles of he-

Table 1. The contribution of the fractions of tetrameric molecules (T), dimers (D) and aggregates (A) in hemoglobin preparations irradiated in the presence of alcohols under N_2O and argon (expressed as percentage and calculated by EMG approximation).

N_2O

alcohol	dose [kGy]	A	T	D
without alcohol	1.28	59.1	26.0	14.9
ethanol	1.70	–	86.6	13.4
	2.55	–	75.9	24.1
1-butanol	1.70	–	72.4	27.6
	2.55	–	60.9	39.4
t-butanol	1.70	–	50.3	49.7
	2.55	–	40.3	57.7

Argon

alcohol	dose [kGy]	A	T	D
without alcohol	1.83	40.2	45.1	14.7
ethanol	1.83	–	77.5	22.5
1-butanol	1.83	–	76.9	23.1
t-butanol	1.83	–	76.1	23.0

Table 2. Values of log $p_{1/2}O_2$ and the Hill "n" parameter for control hemoglobin and deoxy-Hb irradiated under various conditions with a dose of 1 kGy

	N_2O		argon	
scavenger	log $p_{1/2}O_2$	"n"	log $p_{1/2}O_2$	"n"
not irradiated Hb(control)	1.03 ± 0.01	3.03 ± 0.36	1.03 ± 0.01	3.03 ± 0.36
Hb irradiated without scavenger	0.61 ± 0.06	1.54 ± 0.31	0.75 ± 0.02	2.28 ± 0.16
Hb irradiated with:ethanol	0.76 ± 0.06	2.67 ± 0.41	0.85 ± 0.07	2.25 ± 0.35
1-butanol	0.81 ± 0.04	2.15 ± 0.25	0.88 ± 0.07	2.02 ± 0.20
t-butanol	0.38 ± 0.09	1.53 ± 0.42	0.78 ± 0.08	1.65 ± 0.35

log $p_{1/2}O_2$ – log of partial oxygen pressure corresponding to 50% oxygenation of hemoglobin
n – slope of the linear part (20% – 80% oxygenation) of the Hill plot $\log(y/(100-y)) = f(\log pO_2)$, where y – percentage of oxygenated hemoglobin

hemoglobin irradiated in the presence of ethanol, 1-butanol or t-butanol under the atmosphere of N_2O with a dose 1.7 kGy are shown in Fig 2.

In these preparations a large part of protein was present in the dissociated form as dimers (fraction D). The proportion of the dissociated fraction (D) for irradiated preparations under N_2O depended on the kind of used alcohol and increased in the following order: ethanol 13 per cent, 1-butanol 28 per cent and t-butanol 50 per cent for the dose 1.7 kGy (Fig. 2, Tab. 1).

Functional properties of the hemoglobin

In general, all irradiated hemoglobin preparations were characterized by an increased oxygen affinity (decreased log $p_{1/2}O_2$) and decreased co-operativity (decreased "n" parameter), Tab. 2. t-Butanol radicals caused the greatest changes: a very large increase in oxygen affinity (log $p_{1/2}O_2$=0.38 under N_2O) as well as the total disappearance of co-operativity. Changes in the parameters of oxygenation correlated well with the changes in the level of dissociation of hemoglobin tetramers.

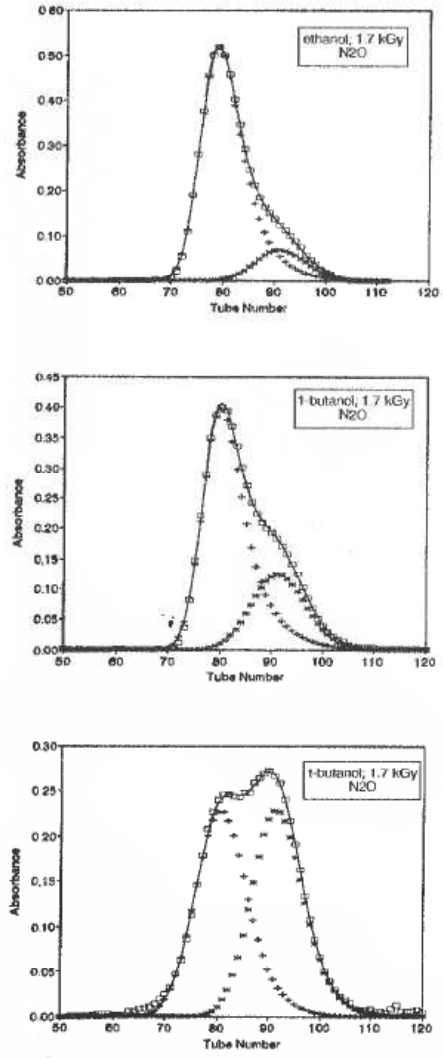


Fig 2. Gel filtration on Sephadex G-100 of hemoglobin solutions irradiated with a dose of 1.7 kGy in the presence of 0.1 mol × dm^{-3} ethanol, 1-butanol and t-butanol under N_2O.

When the preparations were irradiated under the atmosphere of argon no basic differences were observed for individual alcohols in the level of dissociation (Tab. 1) as well as in functional parameters (Tab. 2). This effect is probably caused by recombination reactions of radicals where oxidizing radicals ($^{\cdot}OH$ or t-but$^{\cdot}$) as well as reducing radicals (e^{-}_{aq}) are present.

The destruction of hemoglobin by alcohol radicals is caused, among other factors, by the formation of covalent bonds (cross-linking) between Hb and alcohol molecules (Puchała & Schuessler, 1986) as well as by the H-abstraction from protein by alcohol radicals (i.e. from SH-groups; Puchała, unpublished).

These results indicate that alcohol radicals cause considerable Hb destruction. Most damaging are those radicals in which the unpaired electron is situated on the β-carbon, less damaging are those in which unpaired electron is situated on the β-carbon (ethanol radicals).

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Current Topics in Biophysics, vol. 18(2), 151-154

MECHANISMS OF INDUCTION OF CHROMOSOMAL ABERRATIONS AFTER EXPOSURE TO IONIZING RADIATION

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(Accepted 28 December 1994)

It is often assumed that DNA damage induced by ionizing radiation is expressed as chromosomal aberrations only during the first mitosis which follows after a radiation exposure. While stable-type aberrations, e.g. reciprocal translocations and inversions can be passed to cell progeny, unstable aberrations, like dicentrics or terminal deletions are gradually lost as cells proliferate. This assumption is, on the one hand, based upon results showing a one-to-one correlation between the yield of unstable aberrations and cell survival (Revell, 1983) and, on the other hand, upon estimations of the expected frequency of unstable-type aberration at the second mitosis (Bauchinger *et al.*, 1986). Consequently, while testing for possible chromosome-damaging effects of potential mutagens, great care is taken to score cells at the first mitosis following exposure. There is, however, a growing body of evidence indicating that, at least in some cell systems, a part of the radiation-induced damage is not expressed at the first mitosis but can give rise to novel aberrations several mitotic divisions after the exposure. Thus, a radiation risk assessment based upon the aberration frequency scored at the first mitosis after exposure may actually lead to an underesthmation of the genetic risk induced by the insult. Further, it has been reported by several authors that radiation may lead to a chromosomal instability, resulting in enhanced aberration frequencies several cell generations after the exposure. These findings are interesting as they may lead towards a better understanding of the mechanisms underlying cell transformation.

INTRODUCTION

It is often assumed that DNA damage induced by ionizing radiation is expressed as chromosomal aberration only during the first mitosis which follows after a radiation exposure. While stable-type aberrations, e.g. reciprocal translocations and inversions can be passed to cell progeny, unstable aberrations, like dicentrics or terminal deletions are gradually lost as cells proliferate. This assumption is, on the one hand, based upon results showing a one-to-one correlation between the yield of unstable aberrations and cell survival (Revell, 1983) and, on the other hand, upon estimations of the expected frequency of unstable-type aberrations at the second mitosis (Bauchinger, Schmid & Braselmann, 1986). Consequently, while testing for possible chromosome-damaging effects of potential mutagens, great care is taken to score cells at the first mitosis following exposure. There is, however, a growing body of evidence indicating that, at least in some cell systems, a part of the radiation-induced damage is not expressed at the first mitosis but can give rise to novel aberrations several mitotic divisions after the exposure. Thus, a radiation risk assessment based upon the aberration frequency scored at the mitosis after exposure may actually lead to an underestimation of the true genetic risk induced by the insult.

Further, it has been reported by several authors that radiation may lead to a chromosomal instability, resulting in enhanced aberration frequencies in several cell generations after the exposure. These findings are interesting as they may lead towards a better understanding of the mechanisms underlying cell transformation.

In the following, publications dealing with both aspects of delayed expression of chromosomal aberrations will be briefly reviewed.

DELAYED EXPRESSION OF RADIATION DAMAGE

The analysis of the frequency of chromosomal aberrations in successive mitoses requires either a cell system in which cells divide synchronously, or manipulation of the cell culture conditions allowing to discriminate between cells which passed through different numbers of divisions.

A unique cell system which fulfills the former requirement is the mouse preimplantation embryo, in which the first three cell divisions are highly synchronized. Weissenborn and Streffer (1988) analyzed chromosomal aberrations in three successive mitoses following exposure to various X-

Table 1 Distribution of chromosomal aberrations per embryo in the first to third mitosis (percent) after neutrons (0.25–0.75 Gy) at the 1-cell stage. p.r.: post radiation. Data from Streffer (1993).

	Total aberrations	Chromosome breaks (%)	Chromatin breaks (%)
1st mitosis p.r.	19	33	6
2nd mitosis p.r.	27	16	26
3rd mitosis p.r.	54	51	68

ray or neutron doses at the zygote stage (1 hour post conception). The results indicate that new aberrations are produced after the first postradiation mitosis and expressed during the second and third mitosis. When the number of chromosomal aberrations was calculated per embryo, 40% of all aberrations, 31% of all chromosome breaks and 47% of all chromatin breaks were observed after X-irradiation in third mitosis (Streffer, 1993). The effect was even more pronounced after exposure to neutrons (Table 1). The results of experiments presently carried out at our institute show that treatment of zygotes with AluI, a restriction endonuclease, does not lead to a delayed expression of aberrations indicating that lesions other than double stand breaks, possibly base damage is responsible for phenomenon.

Moore and Bender (1993) also studied X-ray induced chromosomal aberrations in the first and second mitosis of JU56 cell line. Unequivocal second division metaphases were obtained by treating irradiated cells with a low concentration of Colcemid, yielding tetraploid mitoses. The results showed that new chromosomal aberrations can arise during the second post-irradiation interphase. This observation was further confirmed by the fact that cells containing chromosomal aberrations at the first mitosis were not numerous enough to explain lack of clonogenic survival estimated after 10 days of culture.

RADIATION-INDUCED CHROMOSOMAL INSTABILITY

Several authors have found enhanced frequencies of chromosomal aberrations in cells harvested several generations after a radiation exposure. Pampfer and Streffer (1989) irradiated mouse zygotes 1 hour post conception with X-ray, isolated the irradiated fetuses 19 day later (1 day before birth) and cultured fetal fibroblasts *in vitro* for 48 hours. The analysis of chromosomal aberrations yielded a significantly higher level of aberrations in fibroblasts of fetuses irradiated at the zygote stage as compared with controls. An even higher frequency of chromatide-type aberrations was observed in fibroblasts of fetuses carrying radiation-induced gastroschisis, a malformation typical for the mouse strain used. Since the malformation is a consequence of irradiation of the zygote, it most probably results from genomic instability induced by the radiation exposure.

Kadhim *et al.* (Kadhim, Macdonald, Goodhead, 1992) studied chromosomal aberrations in individual colonies of haemopoietic cells derived from irradiated mouse stem cells. Exposure to alpha particles from ^{238}Pu, but not to X-ray produced a high frequency of aberrations in the clonal descendants analyzed after approximately 10–13 cell divisions. The aberrations were mostly of chromatid type and occurred at most in 50% of cells of a given colony. This means that they could not be derived from a single aberration induced in the first interphase by the radiation exposure. There was no evidence to suggest that specific chromosomes were consistently involved in aberrations.

Martins *et al.* (Martins, Sabatier, Ricoul, Pinton & Dutrillaux, 1993) irradiated human fibroblasts with heavy ions (neon, argon and lead) at the 19th passage after initiation of cultures. Cells were harvested approximately at every fifth passage following the irradiation. At the first passage high frequencies of both stable and unstable-type aberrations were observed, the frequency of the latter declining steadily as cells proliferated. Around the 10th passage, the few remaining anomalies were exclusively of the stable type, however, starting approximately at passage 15 a steep increase of cells showing predominantly unstable-type aberrations was observed. The effect was seen for both neon and argon irradiated fibroblasts. In contrast to the data of Kadhim *et al.* (1992), the distribution of aberrations among chromosomes was not random, with chromosomes 1, 13 and 16 being most frequently involved.

In order to study the effects of X-irradiation on clone forming ability and karyotypic abnormalities in human peripheral lymphocytes, Holmberg *et al.* (Holmberg, Fält, Johansson & Lambert, 1993) exposed lymphocytes to 3 Gy of X-rays *in vitro* and analyzed the karyotypes after culturing clones from individual cells for 9–34 days. The results showed that the X-irradiation gave rise to karyotypically abnormal cells which were able to proliferate and form expanding cell clones. Karyotype

abnormalities were found in 65% of irradiated clones. The majority of the abnormal clones showed the same aberrant karyotype in all cells, indicating that it originated during the first interphase after radiation exposure. In some clones, however, cells were found containing novel, nonclonal aberrations which obviously originated during later cell divisions. The increased frequency of such cells was seen only in clones with clonal aberrant karyotypes. This suggests that the delayed expression of chromosomal aberrations is linked to some radiation-induced changes of the karyotype.

The occurrence of enhanced frequencies of chromosomal aberrations several cell generations after an X-ray exposure was also reported by Morgan and Marder (1993). They have applied the technique of chromosome painting to detect chromosomal rearrangements of the human chromosome number 4 in a hamster hybrid cell line. In contrast to the results of Holmberg *et al.* (1993) no clear cut correlation between an abnormal karyotype and chromosomal instability was evident.

CONCLUSION

An increasing body of evidence exists indicating that an exposure to ionizing radiation can lead to a delayed expression of chromosomal aberrations. At least in some cell system, novel aberrations can arise in the second and even third mitosis following exposure. It is not known which kind of DNA damage is responsible for that phenomenon, but the fact that treatment of embryos with a restriction enzyme which induced only double strand breaks does not lead to novel aberrations in the second and third mitosis indicates that damage other than the DNA double strand break is involved.

Several authors have observed a high frequency of novel chromosomal aberrations occurring several cell generations following a radiation exposure. When successive generations of fibroblasts were screened for aberrations, an initial decrease and a subsequent increase of the frequency of aberrant cells was observed (Martins *et al.*, 1993), indicating that the aberrations do not arise from some long lived DNA damage but are rather a result of a genomic instability induced by the radiation exposure. Genetic instability is also thought to be responsible for observed delayed reproductive cell death and delayed mutations (Chang & Little, 1992) in cell surviving an exposure to ionizing radiation. How radiation induces genetic instability is not clear. It is assumed that radiation may lead to a deletion of a gene or set of genes responsible for maintaining genomic integrity. For example, it has been proposed that a defect in cell cycle checkpoint may lead to genomic instability (Hartwell, 1992). Since genomic instability frequently precedes tumorigenesis, futher studies of the phenomenon may lead towards a better understanding of the mechanisms underlying neoplastic transformation.

Acknowledgements

This work was supported by the Bundesministerium für Umwelt, Naturschutz und Reaktorsicherheit, Germany.

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Current Topics in Biophysics, vol. 18(2), 155-157

CELLULAR SOURCES OF HYDROGEN PEROXIDE AND IRON TOXICITY IN YEAST*

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(Accepted 28 December 1994)

The paper presents data showing that ferrous iron toxicity is strictly correlated with availability of glucose as a carbon source and is observed mostly in respiratory competent cells. The toxicity also strongly depends on the phase of growth of yeast cultures. The data suggest that mitochondrial complex III could be one of the main cellular sources of hydrogen peroxide *in vivo*.

INTRODUCTION

Hydroxyl radical $^{\cdot}OH$ is considered one of the two main causative factors of oxygen toxicity (Fridovich, 1975, 1986). Extremely high chemical reactivity of $^{\cdot}OH$ could explain any irreversible damage done to the cell. Hydroxyl radical is formed in the Fenton reaction:

$$Fe^{2+}(Cu^{1+}) + H_2O_2 \rightarrow Fe^{3+}(Cu^{2+}) + {}^{\cdot}OH + OH^- \qquad (1)$$

Availability of both reactants for the reaction is controlled by various cellular mechanisms. It has been assumed that mass formation of hydroxyl radicals *in vitro* could be achieved during ferrous iron overload, fully aerobic conditions being a prerequisite for hydrogen peroxide formation. Under those conditions the amount of hydroxyl radical formed would depend on the rate of hydrogen peroxide generation within the cell. There are numerous potential sources of hydrogen peroxide within the cell. It can be formed directly during some oxidative processes or indirectly from superoxide $O_2^{\cdot-}$ in the reaction catalyzed mainly by superoxide dismutases, present in almost all aerobic organisms.

$$2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2 . \qquad (2)$$

It has been postulated on the basis of in vitro experiments that mitochondria could be the main cellular source of superoxide (Nohl, 1987). Mitochondrial complex III is presumably one of these sources (Boveris, 1978). This postulate is supported by recent finding (Guidot , McCord, Wright & Repine, 1993), that yeast mutant deficient in mitochondrial superoxide dismutase can grow in hyperoxia only in a respiratory deficient form.

Therefore, we have assumed that an impairment of respiratory activity of the cell should strongly influence iron toxicity *in vitro*.

Our preliminary experiments have shown that toxic effects of iron can be observed only when yeast cells are treated with millimolar concentrations of ferrous salts or with molecular iron (carbonyl iron). The use of molecular iron has proven to be difficult under laboratory conditions due to its sedimentation. Therefore, in further experiments ferrous salts were exclusively used.

Experiments performed (Fig. 1) with standard respiratory sufficient ρ^+ and respiratory deficient strains harvested in late exponential phase of growth clearly show that respiratory deficient ρ^- cells are highly resistant to iron overload. Also inhibition of the complex III by antimycin A prevents toxic effects of iron on the standard strain, whereas KCN does not (not shown). The results show also that iron toxicity is observed only in the presence of oxygen, thus confirming involvement of hydroxyl radicals in the process. Respiratory

* This research was supported by Grant 417649101 from the Polish Committee for Scientific Research. Autors are indebted to the Foundation for Development of Polish Science for supplying our laboratoty with necessary equipment.

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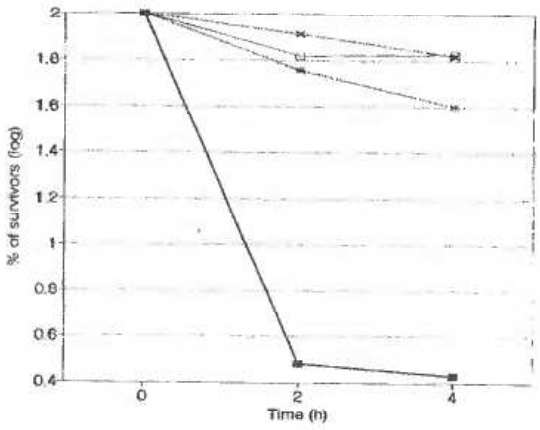

Fig. 1. Cells of the standard respiratory sufficient ρ^+ and respiratory deficient ρ^--strains SP-4 Mat α leu1 arg4, were grown in standard YPGlucose media on rotatory shaker New Brunswick G-24 at 28°C, 150 rev/min under aerobic conditions and harvested in late exponential phase of growth. Cells were centrifuged and suspended in fresh YPGlucose medium. Ferrous salt $FeSO_4(NH_4)2SO_4 \cdot 6H_2O$ was added to the medium and samples were incubated aerobically and anaerobically on a rotatory shaker at 180 rev/min. Aliquots from all samples were taken after different intervals and plated on YPGlucose medium solidified with 2% Difco Agar to establish the number of survivors. Similar procedure was applied to test the role of oxygen in iron toxicity, but suspensions were preincubated during 30 minutes in the atmosphere of nitrogen before ferrous salts were added. Nitrogen was flushed through the suspensions during the experiment. Results are presented as % of survivors. Difco Yeast Extract, Bacto-Peptone and antimycin A produced by Sigma were used. Other chemicals were of laboratory grade.

stars — respiratory sufficient ρ^+

filled squares — respiratory deficient ρ^-

empty squares — respiratory sufficient treated with 10 μg/ml of antimycin A

corsses — respiratory sufficient in the atmosphere of nitrogen

activity of yeast cells changes depending on phase of growth and on the carbon source. Figure 2 shows that standard yeast strain is most sensitive to iron overload in exponential phase of growth, whereas stationary cells are much less sensitive. Standard strain grown on nonfermentable carbon sources tolerates iron. These results show that iron is toxic only for dividing yeast cells grown on glucose as a carbon source. The protective role of antimycin is observed exclusively at the end of exponential phase, when cells switch from fermentative to oxidative metabolism.

It is well known that ferrous iron oxidizes spontaneously in the presence of oxygen. We have determined the rate of disappearance of ferrous iron under experimental conditions (pH 6.0). It appears that within the first hour of incubation 1.5 mM out of 9 mM of ferrous iron was oxidized.

It is worth noting that autoxidation of ferrous cations outside the cell is also a potential source of superoxide. The fact that iron is not toxic for stationary phase and respiratory deficient cells means that amounts of hydrogen peroxide formed in this process are not high enough to be cytotoxic.

In the course of studies on the role of various inhibitors of mitochondrial functions in iron toxicity it has been found that uncouplers, dinitrophenol and CCCP completely prevent iron toxicity, both in exponential and late exponential phase of growth. It suggests that under experimental conditions transport of iron into the cell or within the cell requires a pH gradient.

According to the Fenton reaction only ferric form reacts with hydrogen peroxide to form hydroxyl radicals. On the other hand there is numerous evidence Lesuisse & Labbe (1994) that within the cell amount of ferrous iron is strictly controlled including ferroxidative activity of some proteins. Recently Dancis *et al.* (Dancis, Yuan, Haile, Askwith, Eide, Moehle, Kaplan & Klausner, 1994), have shown that the copper containing protein of yeast cells plays such a role. Mobility of iron within the cell, however, requires reduction of iron, which could be connected also with the activity of ferrireductases and/or the presence of low molecular substances such as ascorbate, glutathione and superoxide. Indirect involvement of superoxide in the formation of hydroxyl radicals is known as metal mediated Haber-Weiss process, which consists of the Fenton reaction (Equation 1) and the following reaction:

$$Fe^{3+} + O_2^{\cdot -} \rightarrow Fe^{2+} + O_2 . \qquad (3)$$

Yeast cells do not possess ascorbate, and thus the role of superoxide in reducing oxidized iron salts could be very important. Therefore, we have tested sensitivity of superoxide dismutase deficient *sod1* mutants to iron (Biliński, Krawiec, Liczmański & Litwińska, 1985). The results have shown that *sod1* mutants in which the level of superoxide is highly elevated Imlay and Fridovich (1991) can grow at 4 mM ferrous iron, whereas the wild type strain tolerates 10 mM iron. The results suggest that indeed the role of superoxide in iron toxicity could be important also *in vitro*.

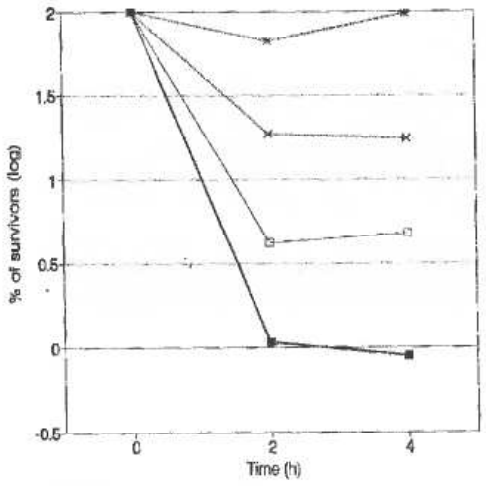

Fig. 2. The procedure was identical to this described in the Legend to Fig. 1, but cells were harvested in different phases of growth or on different sources of carbon.
stars — exponential culture on YPEthanol medium
filled squares — exponential culture on YPGlucose medium
empty squares — late exponential culture on YPGlucose medium
crosses — stationary culture on YPGlucose medium

Our studies on the mechanisms of iron toxicity allow the following conclusions: Ferrous salts are toxic for yeast cells growing on glucose as a carbon source. This toxicity is completely prevented by uncouplers which suggests an involvement of pH gradient in iron uptake into the cell. During transition from fermentative to oxidative metabolism, mitochondrial complex III is presumably the main cellular source of hydrogen peroxide. The other source of hydrogen peroxide, much more powerful, dominates during fermetative phase of growth, but its nature is so far unknown. It could correspond to NADH oxidoreductase of heart mitochondria described by Nohl (1987).

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Current Topics in Biophysics, vol. 18(2), 158-159

OXYGEN EFFECT IN THE RADIOLYSIS OF PROTEINS

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(Accepted 28 December 1994)

If a dilute aqueous solution of proteins is irradiated, proteins are modified by the reactions of OH-radicals and superoxide radicals. Amino acid analyses on irradiated ribonuclease showed a loss of cystine, methionine, tyrosine, phenylalanine, lysine and histidine. The most important reaction induced by radiolysis of proteins in the presence of oxygen is the fragmentation of the peptide chain, while under nitrogen aggregation is dominant. This peptide chain cleavage is not a random process, but leads to specific protein fragments which can be separated by electrophoresis. The estimated molecular weights of these fragments support the assumption that the preferential breaking site is the aminoacylproline peptide group.

INTRODUCTION

Since the total weight of mammalian cells consists of about 70% of water and 20% of different proteins, irradiation of cells induced mainly reactions of these two components. To learn more about this kind of reactions radiolysis of diluted protein solutions has been studied. In that case the interaction of the ionizing radiation is primarily with water. Radiolysis of water yields mainly the three radicals: OH and H-radicals and the hydrated electrons e^-_{aq}. The reactions of all three radicals can only be observed under nitrogen. If oxygen is present, the following reactions occur:

$$e^-_{aq} + O_2 \rightarrow O_2^- + nH_2O$$
$$H^{\cdot} + O_2 \rightarrow HO_2^{\cdot}$$

So if irradiation is carried out under air, only OH-radicals and superoxide radicals could react with the proteins but the $O_2^{\cdot-}$ is rather unreactive.

Mainly two kinds of alterations of proteins were observed after irradiation of protein solutions:

alterations of the amino acid composition;

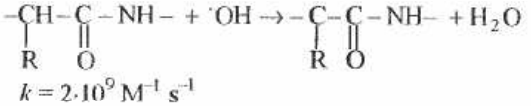
alterations of the molecular size: aggregation and fragmentation.

Alterations of amino acid composition

In general the reactions of OH-radicals with amino acid become only fast, if the side chains contain aromatic residues or sulfur. Only tryptophane, tyrosine, phenylalanine, histidine, arginine, cysteine, cystine and methionine have rate constants for their reactions with OH-radicals which are higher than $10^9\ M^{-1}\cdot s^{-1}$. Hydrated electrons react with a comparable rate constant only with cysteine and cystine. When ribonuclease was irradiated with high doses under different conditions amino acid analyses showed a loss of cysteine, methionine, tyrosine, phenylalanine, lysine and histidine. All these amino acid residues lysine have high rate constants for their reactions with the primary radicals. Similar results were obtained with lactate dehydrogenase.

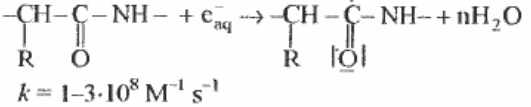
Irradiated ribonuclease was separated by gel filtration in two components, one was monomer and enzymatic active and the other one was inactive and aggregated. Amino acid analyses of both components showed the same loss of amino acid residues. This result gave evidence that the change in amino acid composition did not cause the inactivation of enzyme, but the change of molecular size led to the loss of activity.

Besides the amino acid residues the primary radicals react rather fast with the peptide chains itself. The reaction of OH-radicals leads to a C_α-radical



$k = 2\cdot10^9\ M^{-1}\ s^{-1}$

and the reaction of the hydrated electrons forms a carbon-radical:



$k = 1\text{–}3\cdot10^8\ M^{-1}\ s^{-1}$

Though the reaction rates of amino acid residues and the peptide chain are similar, the scavenger capacity of the peptide chain is at least 3–5 times greater than the one of the tyrosyl residues.

Alterations of the molecular size

When proteins were irradiated under N_2, the main reaction was the aggregation of the molecules. The aggregates were separated by gel filtration or by polyacrylamide gel electrophoresis (PAGE) with and without sodium dodecylsulfate (SDS). Since most aggregates were not destroyed by SDS, new intermolecular covalent bonds must exist after the radiolysis. All different protein radicals can react with each other or they can react with another protein molecule to from covalent bonds.

If radiolysis of proteins is carried out under air, the aggregation process is inhibited, because the protein radicals react very quickly with oxygen to yield protein peroxyl radicals. In the presence of oxygen only OH-radicals react with the proteins and under H-abstraction C_α-radicals in the peptide chain are formed. According to a mechanism published by Garrison (1987) the peroxyl radical derivative can undergo decomposition to form an imino derivative, which hydrolyses spontaneously to yield two peptide fragments, an amide from the N-terminal amino group and a ketoacyl derivative. This mechanism indicates that all C_α-residues have an equal chance to be attacked and peptide chain breaking was expected to be a random process. However, SDS-PAGE of under air irradiated and reduced bovine serum albumin yields a separation of specific protein fragments. Such separation into different distinct fragments could be observed over a large dose range. Since these results show that the radiation induced cleavage of the peptide chain is not a random process, we have to assume that there are sites in the peptide chain which have a higher probability to break than other. The usual peptide bonds are secondary amine groups, only the peptide bond of the aminoacyl-proline is a tertiary amide bond, which is easier to oxidize than the secondary amide bond. Therefore as a possible reaction it is suggested that protein-peroxyl radicals attack the partly positive charged C-atoms before the prolyl residues. This oxidation leads to peptide chain scission before the prolyl residues. This mechanism is hypothetical, but we got very good support for it by comparing the molecular weights of the fragments determined by SDS-PAGE and the molecular weights of the fragments calculated under the assumption of a single break of an aminoacyl-proline bond per protein molecule. There was a very good coincidence in the case of bovine serum albumin where we obtained a separation into 12 fragments. After radiolysis of haemoglobin the densitograms of SDS-PAGE showed a separation into 4 fragments with molecular weights which agreed with our assumption. After radiolysis of ribonuclease in the presence of oxygen 6 fragments could be separated and their molecular weights corresponded to the calculated values.

Furthermore our hypothesis got support by results of Davies & Delsignore (1987). They found that radiation-induced fragmentation of bovine serum albumin produced new carbonyl groups with no apparent increase in free amino groups, which was demonstrated by an assay with fluorescamine. If peptide bond splitting would have been at the usual secondary bond, the reactivity of flourescamine should increase. Since proline does not react with fluorescamine, no increase was found.

In the case of haemoglobin even under anaerobic conditions radiolysis produced fragments, because the haem group and peroxides react with release of oxygen.

Further information on oxygen effect you will find in the following publications. (Jung & Schüßler, 1967, Schüßler, & Jung , 1967, Schüßler & Herget, 1980; Schüßler & Schilling, 1984, Puchala & Schüßler, 1993).

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Current Topics in Biophysics, vol. 18(2), 160-167

EFFECT OF ADRIAMYCIN AND GAMMA RADIATION ON CULTURED MAMMALIAN CELLS MEASURED BY FLOW CYTOMETRY

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The objective of this study was to compare effect of gamma radiation and adriamycin on cultured mammalian cells. Flow cytometry (FCM) technique was applied to study changes in cell structure and conformation of two immortalized Chinese hamster cell lines (CHO ovary and B14 peritoneal fibroblasts). Changes in cell shape and structure as response of cells to damaging effect of radiation and drug were estimated. Cells identification was done on the basis of forward and side scattered light. Damage to cells after treatment with different doses of gamma radiation and concentrations of adriamycin was estimated as the relative percentage of structrally changed cells present in the total population of measured cells. It has been found that gamma radiation as well as adriamycin caused significant dose-dependent changes in cell shape and structure. Both investigated cell lines were different in their sensitivity to radiation and drug although they showed similar distribution of cell cycle. CHO cells were more sensitive to gamma radiation compared to B14 peritoneal fibroblasts, while no differences in sensitivity to adriamycin between the both cell types were found.

INTRODUCTION

Adriamycin (ADR) is a representative member of the anthracycline antibiotic family which like radiation is widely used in the clinical treatment of several human cancers (De Vita, Hellman & Rosenberg, 1989; Chabner, 1992). Both types of agents are also known mutagens and carcinogens (Marquardt, Philips & Sternberg, 1976; Solcia, Ballerini, Bellini, Sala & Betazolli, 1978; Bucciarelli, 1981; Borek, 1983, 1987).

There are currently several hypotheses proposed to explain the biological activity of adriamycin. One of them attributes drug cytotoxicity to the production of reactive free radical species and the ability of these radicals to destroy DNA and cell membrane (Marks & Fox, 1991; Daoud, 1992; Feinstein, Canaani & Weiner, 1993; Krugh, 1994). It has been demonstrated that adriamycin like other quinones with antitumour activity can be reversibly oxidized-reduced in aerobic conditions. This results in the formation of semiquinones and oxygen radicals (Goodman & Hochestein, 1977; Keizer, Pinedo, Schnurhuis & Joenje, 1991; Benchekroun, Sinha & Robert, 1993) in electron-carrying chains in microsomes (Bachur, Gordon & Gee, 1977; Mimnaugh, Gram & Trush, 1983), mitochondria (Davies & Doroshow, 1986) and nuclear fractions of the cells (Bachur, Gee & Freedman, 1982; Mimnaugh, Kennedy & Trush, 1985). Autoxidation of the semiquinone leads in turn to generation of active oxygen species such as superoxide ($O_2^{\cdot}$) and hydroxyl radical ($^{\cdot}OH$) which readily destroy both cell membrane (Daoud, 1992) and DNA (Marks & Fox, 1991; Rowly & Halliwell, 1983; Miura, Muraoka & Ogiso, 1984; Feinstein *et al.*, 1993) via lipid peroxidation (Gutteridge, 1984; Cummings, Bartoszek & Smyth, 1992) and induction of single and double strand breaks (Pommier, Schwartz, Kohn & Zweilling, 1984; Fisher, Brown & Patterson, 1990; Whitaker, 1992). Both the carcinogenic and the therapeutic activities of anthracycline drugs are thought to arise mainly from their DNA modulating action. Adriamycin like other anthracycline drugs interacts with DNA by intercalation (Neidle, 1986; Chaires, Fox, Herrera, Britt & Waring, 1987; Krugh, 1994), groove binding (Leupin *et al.*, 1990), and covalent attachment of the tetracyclic quinone or one of its metabolites (Cummings, *et al.*, 1991; Purewal & Lier, 1993) and its cytotoxicity is largely the result of this binding. It has been shown that drug is capable of inducing condensation of DNA (Kapuszcinski & Darzynkiewicz, 1986; Cera & Palumbo, 1990) and chromatin (Walds & Center, 1981, 1982; Palumbo, Cera, Marciani & Palu,

1988) as well as DNA interstrand and DNA-protein crosslinks (Levin, Silber, Israel, Goldfeder, Khetarpal & Potmesil, 1981; Konopa, 1990; Gullinane, Rosmalen & Phillips, 1994). Another very important mechanism of action of adriamycin and related compounds is inhibition of replication enzymes and topoisomerases (Tanaka & Yoshida, 1980; Kohn, 1983; Tewey, Chen, Nelson & Liu, 1984; Deffie, Batra & Goldenberg, 1989; Beek & Danks, 1991) which results in subsequent blocking of nucleic acids synthesis (Gullinane & Philips, 1990; Ramachandran, Samy, Huang, Yuan & Krishan, 1993).

In this study we use the light scattering FCM method to compare response of two immortalized rodent cell lines (Chinese hamster ovary (CHO) and peritoneal fibroblasts (B14)) to gamma radiation and adriamycin.

MATERIAL AND METHODS

Cell culture

Chinese hamster ovary cells (CHO line) were routinely subcultured in monolayer with Hamm's F12 medium supplemented with 10% foetal calf serum, 4 mM L-glutamine and 5 μg/ml gentamycin. Chinese hamster lung fibroblasts (B14 cell line) were cultured in monolayer with MEM Eagle medium containing 10% heat-inactivated new born calf serum and 5 μg/ml gentamycin. Cells were kept at 37°C in a water-saturated incubator containing 5% CO_2 and 95% air. Prior to experiments cells in exponential growth were plated at 10^5 cells per 60-mm dish and were incubated for 2 days to ensure asynchronous growth.

Irradiation conditions

For irradiation cells were removed from incubator, washed twice with cold PBS to remove the medium and 500 μl of cold PBS were added to each dish.

Cells in monolayer were exposed in sterile conditions to various doses of gamma rays with a ^{60}Co source at a dose rate of 35 kGy/h, in air, at 4°C. Afterwards cells were washed with fresh cold 0.9% NaCl, trypsinized and centrifuged at 1000 rpm for 5 min at 4°C. For flow cytometry analysis 10^5 cells were suspended in 1 ml of PBS and kept on ice until use.

Drug treatment.

Adriamycin was dissolved in deionized water immediately before use. A 3.5 mM stock solution of the drug was added to a desired final concentration and cells were incubated with the drug for 3 hours. Plates were removed from the incubator, cells rinsed twice with 0.9% Nacl to remove the drug, trypsinized, washed twice with 37°C PBS and centrifuged at 1000 rpm for 5 min at 4°C.

Flow cytometry

Flow cytometry (FCM) technique was applied to study changes in cell structure and conformation as well as cell cycle distribution. For estimation of small angle (forward) and large angle (side) light scattering 10^5 cells suspended in PBS were passed through the flow chamber in a flat laminar flow. Flow cytometric scattering patterns were generated using an Argus flow cytometer, (Argus, Oslo, Norway). Histograms were analyzed by Scatron computer programme.

For estimation of cell cycle distribution $1{\cdot}10^6$ cells were suspended in a 10 ml of ice-cold 70% ethanol and placed at –20°C until use. Immediately prior to the cytometric analysis, cells were centrifuged at 1000 rpm at 4°C for 5 min and resuspended in a 0.1% citrate solution containing 2 μg/ml of DAPI (4',6-diamino-2-phenylindole). Flow cytometric patterns were generated using a Partec PAS-II flow cytometer (Particle analyzing system) (PARTEC, Basel, Switzerland) and cell cycle components were identified by computer analysis.

RESULTS AND DISCUSSION

The light scattering of cells is a complex function of their shape, composition and internal structure. The relative contribution of those various cell characteristics vary significantly with the scattering angle. Generally, forward scattering, i.e. scattering at small angles depends primary on cell size, whereas structure becomes relatively more important at larger scattering angles. Thus, flow cytometric measurement of the light scattering of cells at both low and large scattering angles provide an efficient method for differentiating between different cell types. The damaged and dead cells are clearly separated from intact ones by this type of measurement. Their small angle to large angle scattering ratio is being much lower than that of living cells.

In our study changes in cell shape and structure as response of cells to damaging effect of radiation and adriamycin were estimated on the basis of the small to large angles scattering ratio and calculated as the relative percentage of structurally changed cells present in the total populations of measured cells. For FCM analysis the FSC (forward scatter) vs SSC (side scatter) dot and contour plots of all events obtained for each sample were divided into four fixed light scatter sec-

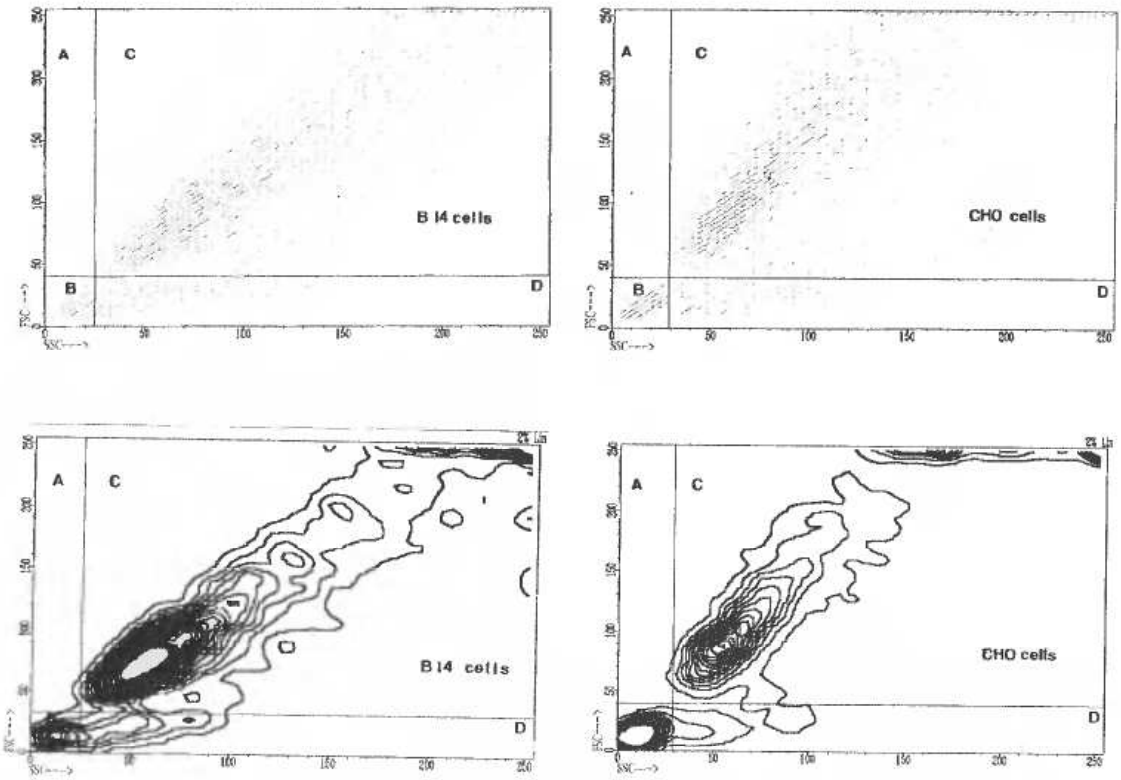

Fig. 1. Forward scatter (FSC) vs.side scatter (SSC) dot and contour plots of cultured Chinese hamster ovary (CHO cell line) and peritoneal (B14 cell line) fibroblasts. Each dot represents one cell. The outermost contour line represents more than 10 cells. The number of cells is doubled each time the contour is narrowed.

Sections:

B – debris and cell aggregates

C – population of intact cells showing high FFC/SSC ratio

D – population of damaged and dead cells showing high FFC/SSC ratio

tions (boxes A, B, C and D). Two different populations, showing different ratio of small/large angle scattering were displayed in all histograms. Examples of scattergrams are shown on Figs 1 and Fig 2. In Fig.1 scattergrams of control cultures are displayed, in Fig.2 – scattergrams of cultures subjected to 10 Gy of gamma radiation or 10 µg adriamycin/ml culture medium. More than 90% of control cells displayed in the box C of histograms had uniform characteristics of high forward scatter and low orthogonal scatter (Fig 1). After irradiation and ADR treatment the same populations of CHO and B14 cells undergo significant changes in scatter characteristics (Fig.2). Box C of the scattergrams reflects the number of intact cells while a box D – damaged and dead cells. In the populations of treated cells the proportions of cells in both sections of histograms (boxes C and D) have changed significantly in a dose dependent manner; the number of cells in a box C (greater FSC/SSC ratio) decreased whereas the number of cells in the box D (lower FSC/SSC ratio) increased. Results are summarized in Tab.1 and Tab. 2 and in Fig.4 and Fig 5.

Both cell lines differ in their sensitivity to gamma radiation. CHO cells were more sensitive compared to B14 peritoneal fibroblasts. Quantification of percentage of damaged cells following exposure to gamma radiation has shown 4-fold increase of populations of damaged cells following 200 Gy of gamma radiation in both cell types. FCM analysis showed that both cell lines are characterized by similar cell cycle distribution (data not shown). No significant differences in the percentage of cells in a particular phase of the cell cycle between the both cell types were found which could in part explain this effect. At the same time both cell lines response in similar way to adriamycin (Tab.2). The observed changes were dose-dependent up to the concentration of 5 µg/ml of the drug. At the greater concentrations of adriamycin (10 and 20 µg/ml) the population of damaged cells remained similar (Tab.2). No statistically significant difference in sensitivity to adriamycin between the two types of cells was found while a statistically significant difference ($p < 0.005$) in the number of damaged cells was observed at all doses of gamma radiation except at

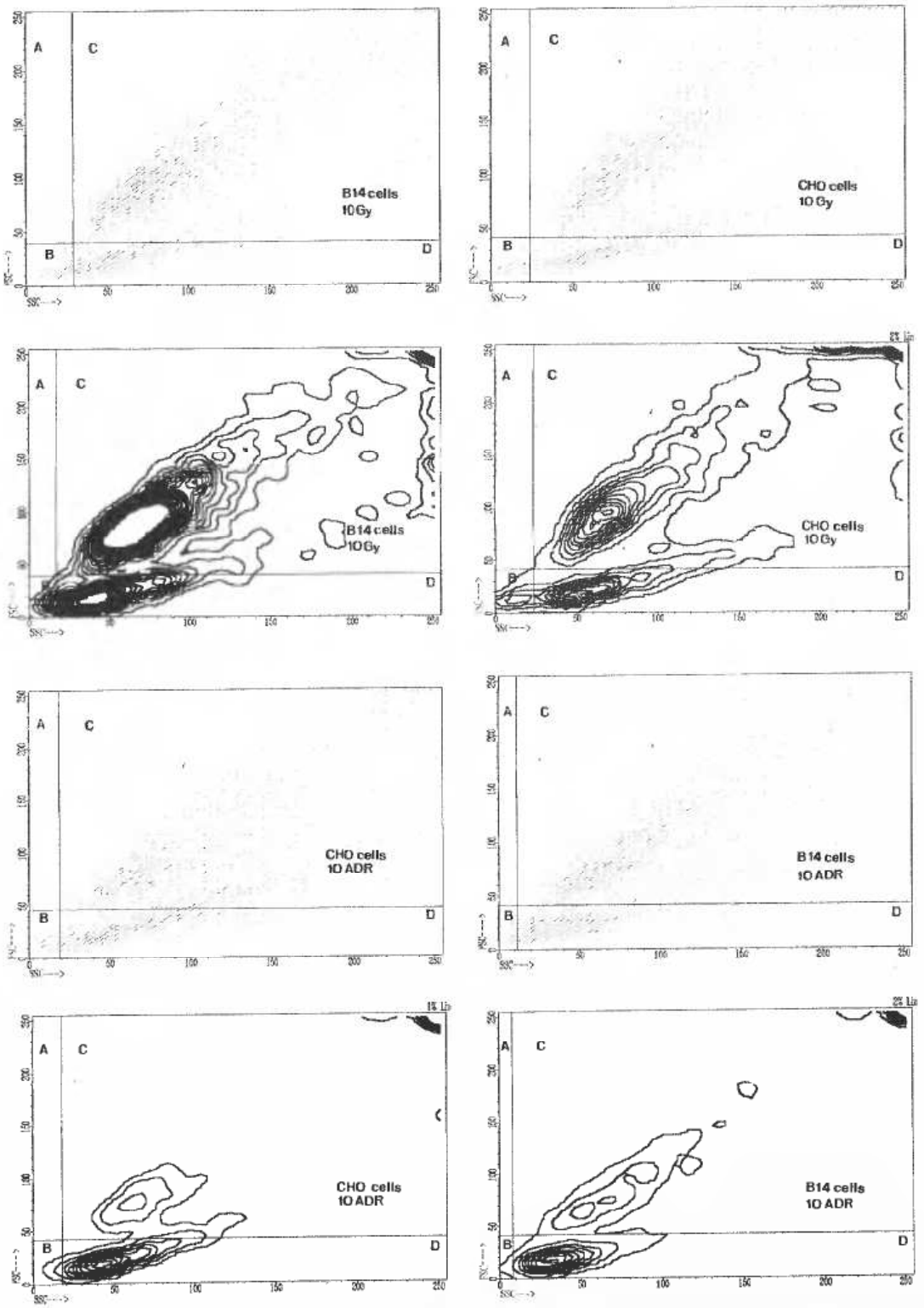

Fig. 2 Changes in scatter characteristics as a result of differences in response of cultured Chinese hamster ovary (CHO) and peritoneal (B14) fibroblasts to 10 Gy of gamma radiation and ADR treatment (10 µg/ml culture medium). Note an increase of population of damaged and dead cells in the section D of the scattergrams.

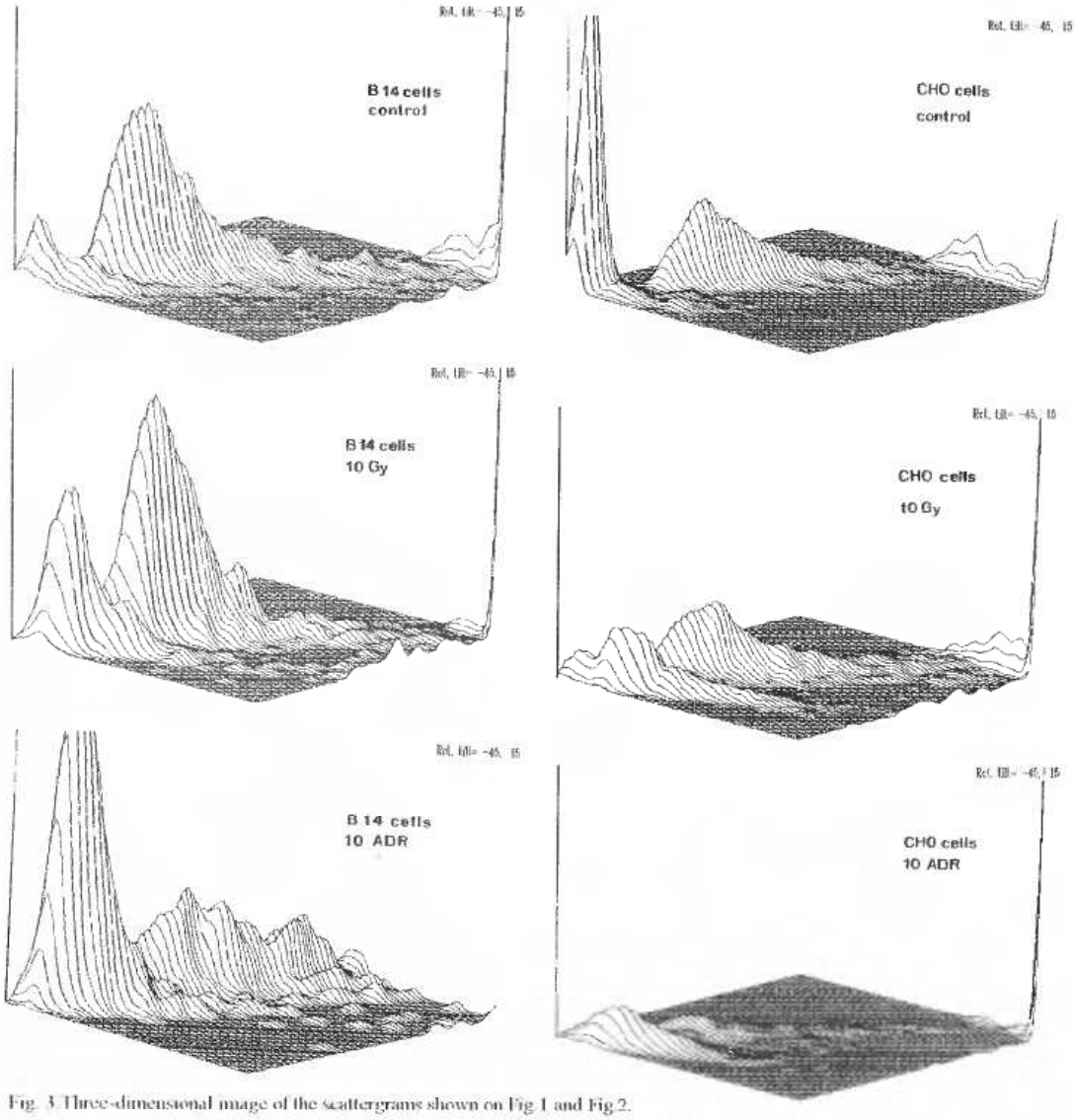

Fig. 3 Three-dimensional image of the scattergrams shown on Fig 1 and Fig 2.

Table 2. Quantification of percentage of damaged cells following treatment withdifferent ncentrations of adriamycin

Adriamycin (μg/ml)	CHO cells mean ± SD[1]	B14 cells mean ± SD	t test[2]
Control[3]	9.95 ± 1.24	11.04 ± 1.21	N.D.[4]
2	28.85 ± 2.42	28.65 ± 2.21	N.D.
5	40.45 ± 3.96	34.69 ± 2.98	N.D.
10	40.79 ± 4.22	39.83 ± 3.66	N.D.
20	40.92 ± 3.76	40.95 ± 3.90	N.D.

[1]Average percentage of damaged cells in total populations of measured cells
[2]Significance was determined using a two-tailed test
[3]No adriamycin treatment
[4]No statistical difference between the two groups

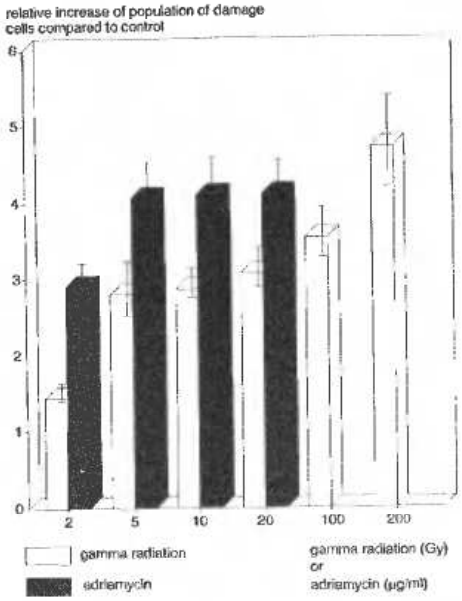

Fig. 4. Canges in cells shape and structure as response to damaging effect of gamma radiation and adriamycin (Chinese hamster ovary fibroblasts – CHO cell line).

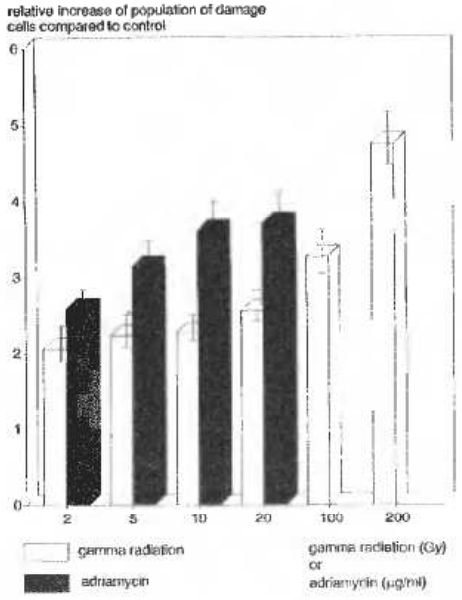

Fig. 5. Canges in cells shape and structure as response to damaging effect of gamma radiation and adriamycin (Chinese hamster peritoneal fibroblasts – B14 cell line).

all doses of gamma radiation except at 200 Gy (Tab.1). Generally, the adriamycin has caused higher relative percentage of damaged cells compared to gamma radiation (Tab.1 and 2, Fig.4 and Fig. 5). Under these conditions cells are given a vast excess of DNA strand breaks (Powell, 1990) which probably covers the differences in their sensitivity observed after lower doses of radiation (Tab.1). Distinct response of cells to radiation and to adriamycin might be considered as the effect of different mechanisms through which both agents act on a living cell. While the ionizing radiation induce mainly DNA strand breaks via oxygen free radical formation in the most cells, the contribution of this type of free radical damage to the cytotoxicity and antitumour effect of adriamycin is probably minor (Whitaker 1992; Sognier *et al.*, 1991).

On the basis of our results we can summarize that the most significant changes in cell structure and shape appeared in the range of the 0–5 µg/ml concentrations of adriamycin. Further increase of drug concentration did not have any effect. Both cell lines showed different sensitivity to gamma radiation but similar response to adriamycin. Chinese hamster ovary fibroblasts were significantly more sensitive to ionizing radiation compared to B14 peritoneal fibroblasts while no differences in sensitivity to adriamycin between the both cell types were found. FCM light scatter dual parameter (FFC and SSC) estimation is very sensitive and powerful method enabling a convenient and fast screening of the damaging effect of radiation and anthracycline drugs on cultured mammalian cells.

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Current Topics in Biophysics, vol. 18(2), 168-171

THE EFFECT OF ALDEHYDIC PRODUCTS OF LIPID PEROXIDATION ON STRUCTURE OF ERYTHROCYTE MEMBRANES

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(Accepted 28 December 1994)

The present study was undertaken to evaluate the effect of the stable end-product of lipid peroxidation – malondialdehyde (MDA) and some other aliphatic aldehydes on the structure and functional properties of human erythrocytes. Modification of erythrocyte membranes by MDA induced quenching of membrane proteins tryptophan fluorescence, destroyed the compact structure of membrane skeleton proteins and decreased the fluidity of a lipid bilayer. Middle and long chain aliphatic aldehydes perturbed the membrane lipid bilayer and disturbed the protein – lipid interactions in the erythrocyte membrane. The effect of aldehydes and their derivatives on the structure of erythrocyte membranes depends on the hydrophobicity and the end-group of an effector molecule, the place of localisation of an effector in the membrane and the way of its interaction with membrane components.

INTRODUCTION

Increased levels of active oxygen species or free radicals create a situation, known as oxidative stress, which leads to a variety of biochemical and physiological lesions often resulting in metabolic impairment and cell death. These highly reactive oxygen intermediates can readily react with various biological macromolecules such as proteins and lipids to cause protein destruction and peroxidation of membrane lipids. At present little is known of the molecular mechanisms by which these active species damage the cell components and by which the cell perceives oxidative insult. Lipid peroxidation, induced in the situation of the oxidative stress, may change a membrane lipid composition and increase the level of highly toxic aldehydes – stable end-products of lipid peroxidation, such as malondialdehyde (MDA) and hydroxyalkenals. The aldehydes, produced in these pathological conditions, may induce posttranslational nonenzymatic chemical modifications of proteins and/or disturbances of membrane lipid bilayers (Esterbauer, Schaur & Zollner, 1991).

The aim of this work was to study the effect of MDA and some other aliphatic aldehydes on the structure and functional properties of human erythrocytes.

Our earlier work showed that the interaction of the long chain aliphatic aldehydes and corresponding fatty acids (the latter can accumulate in the cells as products of endogenous lipases action) with human erythrocyte membranes induced lysis of red blood cells (Zavodnik, Piletskaya & Stepuro, 1991). We assumed that lysis was caused by perturbation of protein-lipid contacts in the membrane by aliphatic aldehydes. The present study showed that the intra- and intercrosslinking of membrane proteins caused by MDA destroyed the compact structure of erythrocyte membrane skeleton proteins and increased microviscosity of the lipid bilayer. Middle and long chain aliphatic aldehydes effectively decreased the bulk lipid and protein-bound lipid microviscosity, perturbed membrane lipid bilayer and disturbed the protein-lipid interactions in the erythrocyte membrane.

MATERIALS AND METHODS

Blood was obtained from healthy donors. Erythrocytes were washed three times in an isotonic NaCl solution and membranes were isolated as was described earlier by Dodge *et al.* (Dodge, Mitchell & Hanahan, 1963). Aliphatic aldehydes and their derivatives (Reakhim, Russia) were added as a concetrated ethanol solution to the membrane suspension immediately before measurements. The ethanol concetration did not exceed 2%. MDA was obtained by the acid hydrolysis of 1,1,3,3-tetramethoxypropane (Aldrich, Germany). Lauric aldehyde was obtained from Ferak (Berlin). The erythrocyte membranes were modified by MDA by incubation of membrane suspensions (2 mg of

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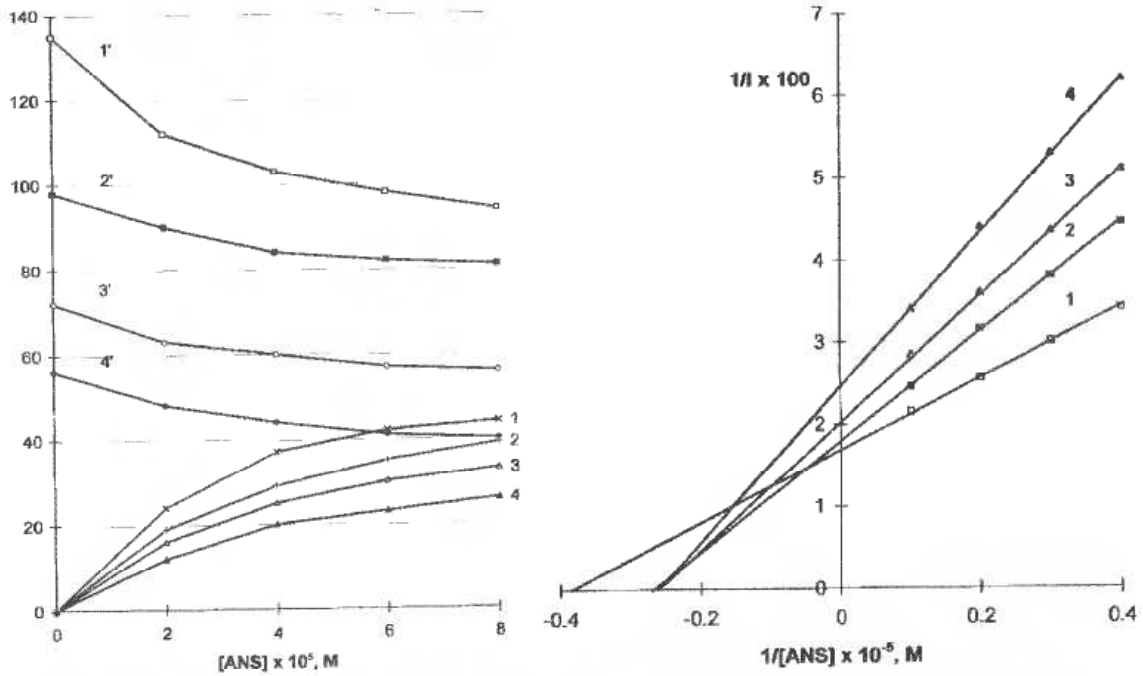

Fig. 1. Interaction of ANS with isolated human erythrocyte membranes: native membranes (1,1'); heated at 37 °C for 2 hours (2,2'); modified in the presence of 2.3 (3,3') and 4.9 mM MDA (4,4'). (A) ANS fluorescence intensity (1–4); membrane protein tryptophanyl fluorescence intensity (1'–4'); (b) reciprocal plot of curves 1–4 from Fig. 1a.

proteins/ml) or intact erythrocyte (1% haematocrit in phosphate-buffered saline, pH 7.4; PBS) with various concentration of MDA at 37°C for 2 hours.

The microviscosity of the membrane lipid phase was measured by the fluorescent method described by Dembo, Glushko and Aberlin (1979). In this method the monomer (395 nm) to excimer (465 nm) fluorescence ratio of pyrene (Sigma, USA) is measured. Isolated membranes were suspended in 0.02 M sodium phosphate buffer, pH 7.4. The protein concentration in the erythrocyte membrane suspension was 0.075 mg/ml and the pyrene concentration in the sample was 5.75 µM. The bulk lipid viscosity was measured by the direct excitation of pyrene fluorescence at 319 nm and the protein-bound lipid viscosity by excitation of pyrene fluorescence at 296 nm due to the energy transfer from tryptophanyl residues of membrane proteins. Pyrene and 1-anilinonaphtalene-8-sulfonic acid (ANS, Sigma, USA) fluorescence spectra were recorded using an Aminco-Bowman spectrofluorimeter (USA). The steady-state fluorescence anisotropy of 1-[4-trimethyl-amino]-phenylhexa-1,3,5-triene (TMA-DPH, Molecular Probes, Eugene, OR, USA), incorporated at the final concentration of $1 \cdot 10^{-6}$ M into the membranes of human erythrocytes of 0.05 haematocrit in PBS, was determined by a Perkin Elmer LS-5B luminescence spectrometer. Polarizers select the vertical and horizontal components of both the excitation (356 nm) and emission (428 nm) beams. The anisotropy was calculated according to Van Blitterswijk, Van Hoeven and Van Der Meer (1981).

The calorimetric curves of the processes of heat denaturation of erythrocyte membranes were registered by precision differential scanning microcalorimeter DASM-1M (Russia). The protein concentration in the membrane suspension was 2.0 mg/ml.

RESULTS AND DISCUSSION

Modification of erythrocyte membranes by MDA induced quenching of membrane protein tryptophan fluorescence accompanied by a short-wave shift (Fig. 1). Keeping the membranes at 37°C for 2 hours without MDA also changed the structure of erythrocyte membranes which was reflected by the quenching of membrane protein tryptophan fluorescence without changing the fluorescence wavelength and by disturbance of the ANS interaction with erythrocyte membranes (a decrease of the ANS association constant with membranes) (Fig. 1).

The short-wave shift of tryptophan fluorescence after membrane modification by MDA may by connected with transfer of some membrane protein tryptophan residues into more hydrophobic regions. Modification of membranes by MDA also decreased the number of the ANS binding sites in

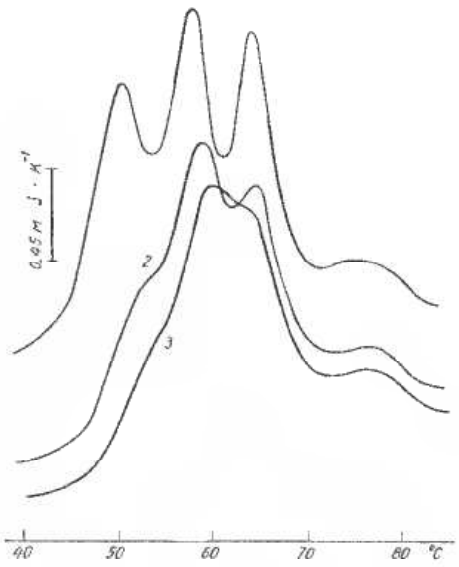

Fig.2. Thermal denaturation of the isolated human erythrocyte membranes (microcalorimetric curves) in the absence of MDA and modified in the presence of 2.3 (2) and 4.9 mM MDA (3). 20 mM sodium-phosphate buffer, pH 7.4.

the membrane without any change of the association constant (Fig. 1).

Fig. 2 shows the result of heat capacity measurements on erythrocyte membranes. Each of the four peaks of denaturation curve of native membranes is believed to be due to a localized thermal transition. The first transition is due to the thermal denaturation of cytoskeleton proteins spectrin and ankyrin (Snow, Vincentelli & Brandts, 1981). The membrane modification by MDA changed the microcalorimetric curves mainly in this temperature region. The analysis of microcalorimetric curves of heat denaturation of membranes after their modification by MDA showed that the denaturation of the cooperative units of membrane proteins occurs in a narrower temperature range as compared to control membranes and the area under the calorimetric curve is smaller (Fig. 2). It is probably due to the disturbance of the compact structure of membrane peripheral proteins, spectrin and ankyrin, and destabilisation of some other proteins by intra- and intercrosslinking of protein molecules.

At the same time, modification of erythrocyte membranes by MDA decreased the fluidity of the membrane lipid bilayer. As shown in Fig. 3 anisotropy values of the TMA-DPH fluorescence increased after incubation of erythrocyte membranes with increasing concentrations of MDA. The decrease of erythrocyte membranes fluidity correlated with the increase of osmotic stability of erythrocytes after modification by MDA (Fig. 3).

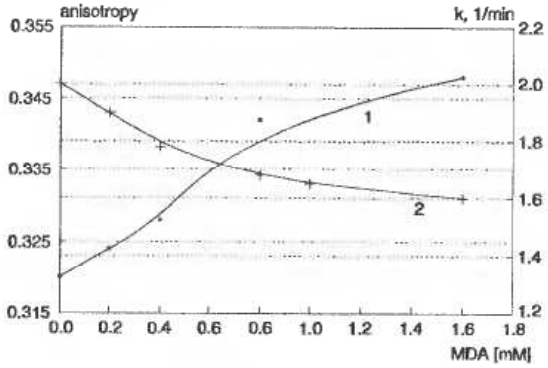

Fig.3. Effect of modification by MDA on the anisotropy of membrane bound TMA-DPH (1) and rate constant of osmotic haemolysis (2) of human erythrocytes.

On the contrary, middle and long chain aliphatic aldehydes and their derivatives increased the fluidity of the membrane. Alteration of lipid microviscosity was estimated by changes of the ratio of fluorescence intensities of the monomeric and excimeric forms of pyrene built into the lipid bilayer. This ratio was proportional to the probe environmental viscosity and inversely proportional to its lateral mobility (Dembo *et al.*, 1979). The presence of long-chain aldehydes and their derivatives decreases the microviscosity of both protein-bound and bulk lipids (Fig. 4), but to the different extent.

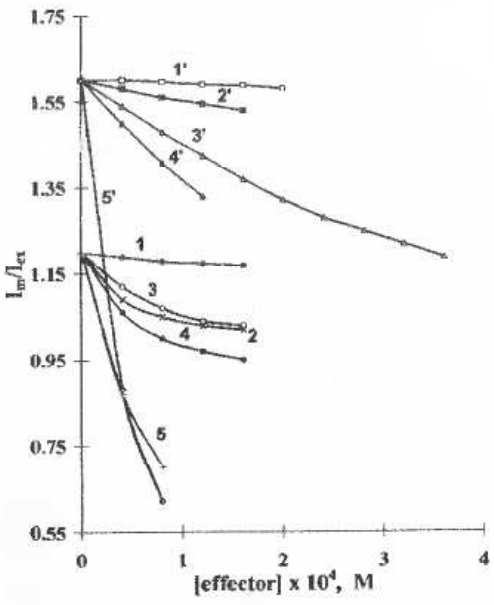

Fig.4. Changes in microviscosity of protein-bound (1–5) and bulk (1'–5') lipids of erythrocyte membranes in the presence of caprylic acid (1,1'), lauric acid (2,2'), lauric aldehyde (3,3'), palmitic acid (4,4'), methyl ester of palmitic acid (5,5'). 0.02 M sodium- phospate buffer, pH 7.4.

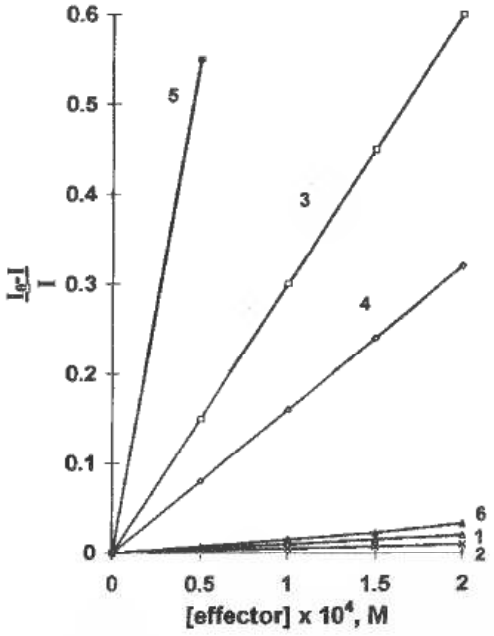

Fig.5. Stern-Volmer plots for the quenching of membrane-bound ANS fluorescence by caprylic acid (1), caprylic aldehyde (2), lauric acid (3), lauric aldehyde (4), palmitic acid (5), methyl ester of palmitic acid (6).

The effect of fatty acids or aldehydes increases with the elongation of the aliphatic chain in the effector molecule (Fig. 4). Aldehydes diminish the bulk lipid microviscosity to a greater extent as compared to long-chain fatty acids. Methyl esters of fatty acids have the biggest effect on lipid microviscosity, the microviscosity of free lipids being decreased to a greater extend (Fig. 4). One may suggest that this effect is related to the better solubility of esters in the lipid phase of the membrane in comparison to the corresponding fatty acids and aldehydes.

Free aldehydes and corresponding free long-chain fatty acids quenched effectively the fluorescence of membrane-bound ANS (Fig. 5). The efficiency of quenching increased as the carbon chain length of aldehydes increased and depended on the end group in the effector molecule (increased in the series of methyl ester of fatty acids < aldehyde < fatty acid).

At the same time aliphatic aldehydes and corresponding free fatty acids decreased the stability of membrane proteins only to a low extent.

It can be concluded that middle and long chain aliphatic aldehydes and their derivatives perturbed the membrane lipid bilayer and disturbed the protein-lipid interactions in the erythrocyte membrane. Aliphatic aldehydes and their derivatives may compete with endogenous phospholipid headgroups for binding with membrane proteins at the lipid-water interface, thus inducing a membrane structure changes. In this situation an effective decrease of the bulk lipid and protein-bound lipid microviscosity, an effective quenching of membrane bound ANS fluorescence as well as lysis of erythrocytes are observed. Modification of erythrocyte membranes by MDA induced quenching of membrane protein tryptophan fluorescence, destroyed the compact structure of membrane skeleton proteins and decreased the number of binding sites for a fluorescent probe ANS. Aldehydes are accumulated in the membrane as products of lipid peroxidation and fatty acids as products of action of endogenous lipases. They affect the arrangement of the lipid bilayer and proteins incorporated in the bilayer and may be factors of cell ageing.

The effect of aldehydes and their derivatives on the structure of erythrocyte membranes depends on the hydrophobicity and the end-group of an effector molecule, the localisation of an effector in the membrane and a way of its interaction with membrane components.

Acknowledgements

This study was supported by the European Fellowship Fund (Institute of Biochemistry and Biophysics, Polish Academy of Sciences) and by the Committee for Scientific Research (Grant No. 6P207 126 04).

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Current Topics in Biophysics, vol. 18(2), 172-175

THE ROLE OF BETA-CAROTENE, VITAMIN C AND E IN THE PROCESSES OF PEROXIDATION IN THE ORGANISM IN EXPERIMENTAL HYPERCHOLESTEROLAEMIA

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(Accepted 28 December 1994)

The study was carried out on guinea pigs receiving during 12 weeks a diet with addition of cholesterol 0.2%. A part of the animals were given with the diet vitamin C and E and/or beta-carotene. It was found that addition of beta-carotene to the atherogenic diet prevented in the same degree accumulation of lipid peroxides in the serum of the animals with hypercholesteroaemia as supplementation of the diet with all three antioxidative vitamins. The addition of 0.2% cholesterol to the diet decreased SOD and CAT activity. The studied antioxidative vitamins restored the activity of these enzymes in a similar degree. The diet with added cholesterol significantly decreased the serum level of GSH, while supplementation of the diet with beta-carotene or vitami C and E caused no change of GSH in the blood of the animals on the atherogenic diet. The obtained results show that addition of beta-carotene or all three studied vitamins given jointly inhibited to a similar extent the accumulation of lipid peroxidation products and restored the activity of antioxidative enzymes which is decreased in experimental hypercholesterolaemia.

INTRODUCTION

The development and progression of atherosclerosis depend on many factors. Studies of humans and experiments on animals show that, besides hyperlipidaemia, an important role is played by peroxidation of lipids in cell membranes and plasma lipoproteins (Steinbrecher, 1980). Ever more frequently the opinion is expressed that accumulation of cholesterol esters in arterial wall cells is preceded by peroxidation of plasma lipids, which facilitates the modification of the native LDL forms (Steinberg, Parthasarathy, Carew, Khoo & Witztum, 1989; Avogaro, Bittolo Bon & Carzolato, 1988). The susceptibility of plasma lipids and lipoproteins to oxidative modification is increased by deficiency if certain vitamins acting as natural antioxidants (Esterbauer, Dieber-Rothender, Striegl & Waey, 1991).

The observation that dietary supplements of vitamins C and E or treatment with them reduces the intensity of peroxidation processes in the organism has been well documented (Packer, 1991; Di Mascio, Murphy & Sies, 1991; Mickle, 1991). However, in recent years it was shown that besides ascorbic acid (vitamin C) and tocopherol (vitamin E) a powerful antioxidative effect was exerted by beta-carotene (Burton, 1989; Gaziano, Manson, Ridker, Buring & Hennekens, 1990; Salonen, Salonen & Nyssänen, 1992). The administration of that vitamin to patients with coronary heart disease (CHD) decreased the anginal symptoms (Gaziano *et al.*, 1990). In animal experiments it was shown that beta-carotene prevented the development of cholesterol deposits in guinea pig aorta during atherogenic diet (Ziemlański, Paczenko-Kresowska, Bukowska & Naruszewicz, 1993).

It has been demonstrated in CHD that the activity of antioxidative enzymes is decreased (Loeper, Goy, Bedn & Rosensztajn, 1987; Panczenko-Kresowska, Ziemlański, Rudnicki & Ruciński, 1992). In experimental atherosclerosis in guinea pigs the activity of superoxide dismutase (SOD) and catalase (CAT) was found to be reduced considerably (Ziemlański *et al.*, 1993).

The purpose of the present study was an assessment of the growing intensity of peroxidation processes during experimental hypercholesterolaemia, and comparison of the antioxidant power of beta-carotene with that of vitamin C and E during experimental atherosclerosis.

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MATERIAL AND METHODS

The experiment was carried out on 50 guinea pigs with initial body weight of 320 g. The animals were divided into two groups: one received diet enriched with cholesterol (0.2 g/100 g of food). In this group three subgroups were isolated: in the first subgroup the animals received the diet with 0.2% cholesterol, in the second this diet was given with added beta-carotene 50 mg/100 g of diet, in the third vitamin C 500 mg/100 g, vitamin E 10 mg/100 g and beta-carotene 50 mg/100 g were added to the cholesterol-enriched diet. The second group served as control, and it was divided into two subgroups: the first one received food with added vitamins, the second one was given the LSK food.

Food and water were accessible *ad libitum* to the animals.

The experiment lasted 12 weeks. After that time the animals were killed by decapitation. In the plasma the amount was determined of substances reacting with thiobarbituric acid (TBA-RS) as an indication of the concentration of lipid peroxides. The Buege and Aust (1978) spectrophotometric method was used and the value of TBA-RS was expressed in nmol/ml.

In erythrocyte haemolysates the activity of superoxide dismutase (SOD, E.C. 1.15.1.1) was determined by the adrenochrome method of Misra and Fridovich (1972) and expressed in activity units U/g Hb. The activity of catalase (CAT, E.C. 1.11.1.6) in the haemolysates was measured by Beers and Sizer method (1952) and expressed in Bergmayer units UB/g Hb.

Reduced glutathione (GSH) was determined in whole blood haemolysates by Beutler *et al.* method (Beutler, Duron & Mikus, 1963) and calculated per mg of haemoglobin.

The obtained results were verified statistically by the Student's t test for paired variables.

RESULTS

Table 1 presents the values of TBA-RS in plasma and the activity of SOD, CAT and GSH in blood haemolysates.

A significant increase was found of TBA-RS level in the blood of the animals on cholesterol-enriched diet. The addition of beta-carotene to this diet inhibited the rise of blood TBA-RS to the same degree as the addition of all three antioxidative vitamins.

Addition of 0.2% of cholesterol to the diet caused a statistically significant (by 28%) decrease of SOD and CAT activity in the blood of the experimental animals. Beta-carotene added to the cholesterol-enriched diet restored SOD activity in about 85%, and supplementation of the diet with all three antioxidative vitamins increased by 96% the SOD activity depressed by cholesterol. CAT activity was restored to about 80% by beta-carotene added to the atherogenic diet.

In the group of animals on the cholesterol-enriched diet the value of GSH in blood was significantly decreased. The addition of beta-carotene or all three vitamins had no effect on GSH level in the blood of the animals with hypercholesterolaemia.

DISCUSSION

It was shown *in vitro* and *in vivo* that as a result of atheromatous lesions developing in the organism active forms of oxygen appear (Gey, 1986). In cells the antioxidative actions are carried out mainly by SOD, CAT and GSHPx enzymes. Another group of compounds present in the cells as well as in systemic fluids are antioxidative vitamins, mainly tocopherols, carotenes, and ascorbic acid.

In the experiments on animals and studies of humans carried out as yet long-term deficiency of antioxidative vitamins was found to lead to athe-

Table 1. TBA-RS level and the activity of antioxidative enzymes in the blood of guinea pigs fed atherogenic diet supplemented with beta-carotene and vitamins C and E.

	Cholesterol-rich diet groups			Control groups	
	cholesterol 0.2%	β-carotene + cholesterol	β-carotene + vitamin C + E + cholesterol	LSK food	β-carotene + vitamin C + E + LSK food
TBA-RS nmole/ml	6.9 ± 0.6*	5.8 ± 0.3	5.9 ± 0.5	5.8 ± 0.6	5.9 ± 0.3
SOD U/g Hb	1192 ± 230*	1408 ± 180	1598 ± 116	1657 ± 248	1747 ± 296
CAT UB/g Hb	33.6 ± 6.1*	42.6 ± 7.6	39.9 ± 9.3	52.9 ± 15.1	48.3 ± 12.7
GSH μg/mg Hb	4.2 ± 0.5*	4.5 ± 0.2	4.5 ± 0.4	5.2 ± 0.7	5.1 ± 0.6

* – $p < 0.05$ in relation to control group (only LSK food)

rosclerotic-like lesions in arterial walls (Kok, Bruijn & Verneeren, 1987; Riemerson, Wood, Macintyre, Elton & Oliwer., 1991). These changes may be reversed by proper diet or vitamin supplementation (Packer, 1991; Mickle, 1992; Ziemlański, Wartanowicz, Potrzebnicka & Paczenko-Kresowska, 1989).

Our earlier works (Ziemlański *et al.*, 1993) and the present study have shown that beta-carotene, similarly as vitamins C and E, is a powerful antioxidant during hypercholesterolaemia, as evidenced by significant prevention of accumulation of lipid peroxides in the plasma in animals with experimental atherosclerosis. In histological examinations Ziemlański *et al.* (1993) found that beta-carotene inhibited the accumulation of atheromatous lesions in guinea pigs on cholesterol-enriched diet.

In the present work we observed an interesting "restoration" of SOD and CAT activity by beta-carotene and vitamins C and E in animals on cholesterol-enriched diet. These results confirmed earlier observations in which it was demonstrated that the activity of antioxidative enzymes previously inhibited by cholesterol was rising with increasing doses of supplemented beta-carotene (Ziemlański *et al.*, 1993).

The exhaustion of GSH, which is a cofactor of glutathione peroxidase, weakens the barrier protecting the enzymes from the destructive effects of oxygen in the cells (Gibson, Hawrylko & McCay, 1985). In the present study the results suggested a decrease of blood GSH level in experimental hypercholesterolaemia. The addition to antioxidative vitamins in this experimental model has not supported the maintenance of an equilibrium between the cofactor and the enzymes.

In summary, it may be said that in the described experimental model the efficiency of beta-carotene as an antioxidant was equal to that of alpha-tocopherol and ascorbic acid.

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Current Topics in Biophysics, vol 18(2), 176-179

THE EFFICACY OF ENDOGENOUS AND EXOGENOUS ANTIOXIDANT PROTECTION OF ERYTHROCYTES AGAINST THE ACTION OF RADICAL GENERATING FACTORS („AGEING *IN VITRO*")

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(Accepted 28 December 1994)

The purpose of our work was to determine the efficacy of antioxidant influence of thiol compounds as well as of vitamin E and acetylsalicylic acid on erythrocytes exposed to radical-generative factors. The level of reduced glutathione in erythrocytes was considered the main determinant of the efficacy of the intracellular antioxidant mechanisms. The endogenic thiol antioxidants prevented first of all the irrivocable changes of hemoglobin (the formation of Heinz bodies) in erythrocytes caused by the prooxidation factor ("ageing *in vitro*"). In a lesser degree they affected the maintainance of the glutathione level in the cell, except GSH + vitamin E. The best protective effect for hemoglobin (expressed in the decreased level of methemoglobin and denaturated hemoglobin) in erythrocytes submitted to prooxidation was achieved by administrating thiourea, an exogenic thiol antioxidant.

INTRODUCTION

Erythrocytes are often affected by prooxidants eg. drugs, and processes such as ageing. These cause the liberation of free radicals, for the inactivating of which the antioxidant protective system of the cells proves insufficient. It is a fact known from literature that thiol compounds which are important components of the cell administered from outside prove antioxidative (Krinsky, 1992; Mascio *et al.*, 1991).

In the red blood cells (as well as in other cells) there exists an antioxidative protective system, which prevents prooxidative processes. To this system belong thiol compounds such as glutathione and its precursor – cysteine, which take part in the scavenging of free radicals by means of enzymatic reactions (Mascio *et al.*, 1991). This system protects hemoglobin from damage and prevents oxidation of the unsaturated fatty acids in the cell membrane as well as its disorganization. The processes of ageing are one of the cases when the cell loses its antioxidative protection. According to Harman, the reason that ageing takes place are the non-balanced radical-generating processes, which may take place both *in vivo* and *in vitro* (Harman, 1992).

The purpose of our work was to determine the efficacy of the antioxidative influence of thiol compounds as well as of vitamin E and acetylsalicylic acid on erythrocytes exposed to radical-generative factors *in vitro* (Gaczyńska, Bartosz & rosin, 1989).

Thiol compounds such as glutathione and its precursor – N-acetylcysteine (NAC), and exogenic thiourea were used as antioxidants. By using glutathione or N-acetylcysteine with vitamin E, a well known and widely used stabilizer of cystoplasmic membranes (Urano, 1990) the possibility of supplementing the erythrocyte antioxidative system under the influence of the prooxidative factor has been tested. The endogenous thiol compounds have been used because, according to current literature, the sulphhydryl groups belong to factors indispensible for the maintainance of the function and the integrity of the cell structure, although the mechanism of these dependencies is not yet fully known (Reed, Pascoe & Craig, 1990; Yang, 1993). The influence of commonly used aspirin (acetylsalicylic acid), which is thought to be thoroughly tested, is still a subject of analysis, among others for its influence on erythrocytes (Watala & Gwoździński, 1993).

The level of reduced GSH has been considered the main determinant of the efficiency of the intracellular antioxidative mechanisms, because it plays a key part in the antioxidative system of the

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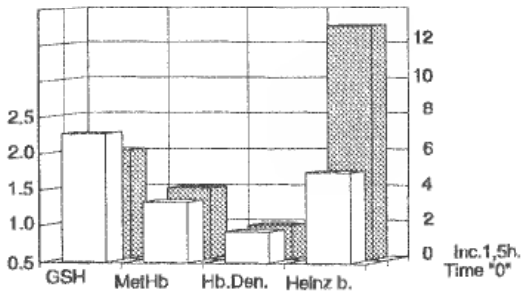

Fig. 1. The effects of prooxidation ("ageing *in vitro*") on human erythrocytes:
GSH – glutatione level (mM);
MetHb – Methemoglobin (index = A 630/542 × 100);
Hb.Den – Denatured hemoglobin (index = A 700/542 · 100);
Heinz b. – per cent of erytrocytes with Heinz bodies (on the right)

erythrocytes. This was confirmed by the results of our previous tests (Woźniak & Grabarczyk, 1992). The changes in erythrocytes caused by the prooxidative factor were assesed by means of indicators, which characterized the cytoplasm (the GSH level, the methemoglobin level, denatured hemoglobin level including Heinz bodies) and the erythrocyte membrane (osmotic resistance, sensitivity to a non-ionic detergent, Tween 20%, and acetylcholinesterase activity).

MATERIAL AND METHODS

Red blood cells were isolated from the whole blood of donors collected into heparin. Erythrocytes were washed to eliminate the effect of serum and leukocytic enzymes and then suspended in PBS to obtain the concentration of $5.0 \cdot 10^8$ cells per ml. The incubation of the erythrocyte suspension for 1.5 hours at the temperature of ± 37°C in 5% CO_2 was introduced as the prooxidative factor. At the same time and under the same conditions, the red cells suspensions were incubated together with compounds, the antioxidative influence of which has been tested, i.e. with N-acetylcysteine (Sigma) (0.1 mM), with N-acetylcysteine 0.1 mM) together with vitamin E (60 µg/ml), with glutathione (Sigma) (1 mM), with glutathione (1 mM) together with vitamin E (Polfa) (60 µg/ml), with thiourea (0.001 mM) as well as with acetylsalicylic acid (0.01 mM). After the incubation of erythrocytes the following parameters were determined:

Membrane parameters: tests for the stability of erythrocyte membranes, i.e. osmotic resistance according to Seeman & Weinstein; sensitivity to 7% Tween 20 according to Hamada & Matsumoto; the acetylcholinesterase level in erythrocytes according to Ellman as described in the previous work (Woźniak & Grabarczyk, 1992).

Cytoplasmic parameters: methemoglobin level (wavelength of 630 nm) and denatured hemoglo-

Table 1. The protective effect of antioxidants on human erythrocytes during "ageing *in vitro*".

	Parameters of erytrocytes	GSH mM/l x ± SD	Methemoglobin index x ± SD	Denatured hemoglobin index x ± SD	Erythrocytes with Heinz bodies (%) x ± SD
	experimental groups				
1	prooxidation n	2.02 ± 0.23 10	1.32 ± 0.19 10	62 ± 0.1 10	13.0 ± 2.2 10
2	glutathione, GSH (1mM) n	2.11 ± 0.24 8	1.26 ± 0.15 8	0.63 ± 0.11 10	9.0 ± 2.0* 9
3	GSH + Vit. E (1mM + 60 µg/ml) n	2.20 ± 0.13* 10	1.39 ± 0.11 8	0.68 ± 0.11 10	10.0 ± 4.0 8
4	N-acetylcysteine (0.1mM) n	2.13 ± 0.25 13	1.22 ± 0.15 14	0.60 ± 0.08 14	8.0 ± 2.0*** 13
5	NAC + Vit. E (0.1mM + 60 µg/ml) n	2.15 ± 0.20 10	1.29 ± 0.08 8	0.68 ± 0.10 10	7.0 ± 3.0*** 9
6	Thiourea (0.001mM) n	1.88 ± 0.33 5	1.12 ± 0.06* 5	0.51 ± 0.04* 5	10.7 ± 3.5 5
7	Aspirin (0.01mM) n	2.21 ± 0.37* 4	1.01 ± 0.12** 4	0.49 ± 0.11 4	20.0 ± 13.0 4

Statistical significance in relation to human erythrocytes "ageing *in vitro*" (prooxidation) without protection: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

bin level (wavelength of 700 nm) by means of routine spectrophotometric method (expressed as relative values to oxyhemoglobin [wavelength of 542 nm] adjusted to value of 1.0), the number of erythrocytes with Heinz bodies expressed in % according to Manoji as well as GSH (reduced glutathione) level determined according to Beutler, as in the paper of Woźniak and Grabarczyk (1992).

The tests were performed on erythrocytes of the peripheral blood of 20 donors. The statistical conclusions were drawn on the basis of the t-Student test. The results were given as $x \pm SD$.

RESULTS

Prooxidation

The introduction of the prooxidative factor (incubation which imitated the process of ageing) caused a significant 15% decrease of the GSH level ($p < 0.05$). This change was accompanied by: a highly significant increase of the methemoglobin level and the percent of erythrocytes with Heinz bodies as compared to the control values (Table 1, Fig. 1). However, the indicators of the stability of erythrocyte membranes did not change significantly under the influence of the radical-generating factor and therefore no further tests on the effect of antioxidants on the erythrocyte membrane were performed.

Prevention of the results of prooxidation

It has been determined that in the employed prooxidation model, endogenous thiol antioxidants have a protective effect on erythrocytes. This was expressed in the increased level of GSH in the tested erythrocytes. However, only the combining of GSH with vitamin E resulted in a significant increase of glutathione content in the red blood cells ($p < 0.05$). The use of endogenous thiol antioxidants also played a significant role in the prevention of the formation of Heinz bodies ($p<0.001$), except GSH with vitamin E. Thiourea, an exogenous thiol antioxidant, helped to reduce significantly the level of soluble hemoglobin, changed during the prooxidation process, unlike the endogenous thiol compounds (GSH, NAC), which did not have a unequivocal protective effect on this form of hemoglobin (Table 1, Fig. 2).

The use of acetylsalicylic acid proved that this compound can prevent both the reduction of the GSH level as well as the increase of the methemoglobin level in the processes of prooxidation of erythrocytes but it does not protect them from the formation of hemoglobin in denatured form or the Heinz bodies (Table 1, Fig. 2).

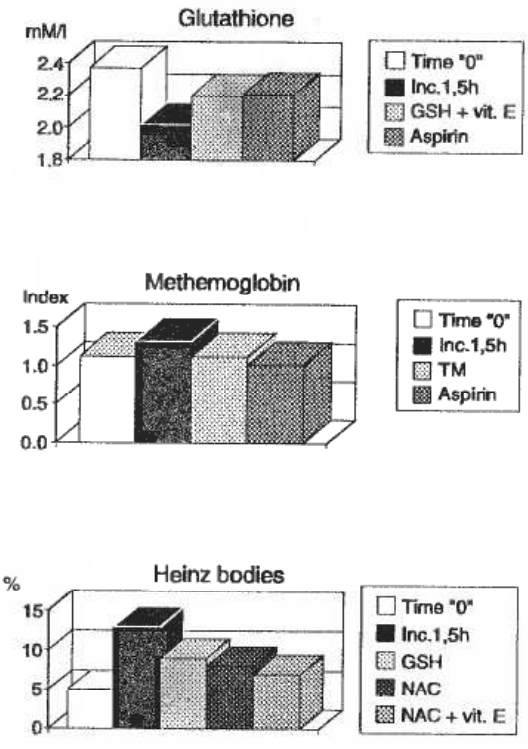

Fig. 2. Protective effects of some antioxidants on "ageing" of human erythrocytes *in vitro* measured by glutathione level, methemoglobin index and percent of erythrocytes with Heinz bodies.

These results are only preliminary and they need to be confirmed in the course of further studies.

DISCUSSION

The prooxidative factor used by us caused significant damage, it was therefore possible to determine the efficacy of factors preventing the results of prooxidation.

The protection against the damaging effects of free radicals may take the form of prevention, counteraction or repair (Sies, 1993). Therefore, in order to protect the erythrocyte from the effects of prooxidation and to strengthen the antioxidation system, endogenous and exogenous thiol compounds were used *in vitro*.

The results obtained show a very selective effect of the antioxidants used in relation to the erythrocyte structures, damaged by the prooxidation process in the specific experimental conditions, although the major protective effect obtained is consistent with the results presented in literature (Chow, 1992; Krinsky, 1992; Selig *et al.*, 1993).

The results of our experiments show that the best effect of protecting the glutathione in an erythrocyte is achieved after administrating GSH together with vitamin E. The effective protection of hemoglobin, on the other hand, could be achieved by administrating thiourea, an exogenous thiol antioxidant. An effective protection against the formation of Heinz bodies (the irreversible changes in hemoglobin) was achieved by administrating glutathione as well as N-acetylcysteine (the precursor of glutathione) together with Vitamin E.

The use of aspirin (acetylsalicylic acid) prevented the reduction of glutathione level and protected hemoglobin against the accumulation of methemoglobin, which indicates that this substance has a very good protective effect in the first stages of the denaturation of hemoglobin. An interpretation of the obtained results would however be premature. These results are preliminary and require confirmation.

CONCLUSIONS

It was determined that, by choosing the "*in vitro* ageing" as a model of prooxidation, the antioxidant system of the erythrocyte could be strengthened by using endogenous and exogenous antioxidants *in vitro*.

The endogenous thiol antioxidants prevented, first of all, the irreversible changes of hemoglobin (the formation of Heinz bodies) in erythrocytes caused by the prooxidation factor *in vitro*. To a lesser degree they affected the maintainance of the glutathione level in the cell, except GSH + vitamin E.

The best protective effect on hemoglobin (expressed in the decreased level of methemoglobin and denatured hemoglobin) in erythrocytes submitted to prooxidation was achieved by administrating thiourea, an exogenous thiol antioxidant.

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Current Topics in Biophysics, vol. 18(2), 180-183

REACTIONS OF OXYGEN RADICALS WITH HEME PEROXIDASES

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By means of pulse radiolysis technique, reactions of oxygen radicals ($OH^{\cdot}$ and $O_2^{\cdot-}$) with horseradish peroxidase (HRP) and lactoperoxidase (LPO) have been studied. The formation of compounds I, followed by their reduction to compounds II, was observed under conditions where hydroxyl radicals were generated in excess. Reactions of superoxide anion with preformed compound II of peroxidases lead to the formation of the new enzyme derivative.

INTRODUCTION

One of the most consistent sources of oxygen radicals in living organisms is the mitochondrial respiratory chain. There, a small proportion (1–5%) of the oxygen consumption undergoes stepwise reduction according to the following mechanism:

$$O_2 \xrightarrow{e^-} O_2^{\cdot-} \xrightarrow{e^-,\,2H^+} H_2O_2 \rightarrow$$
$$\xrightarrow{e^-,\,H^+} 2OH^{\cdot} \xrightarrow{e^-,\,2H^+} 2H_2O$$

The reactive oxygen intermediates $O_2^{\cdot-}$, H_2O_2, $OH^{\cdot}$, are often called activated oxygen species (AOS). Although various physiological functions have been described for AOS in the cell (Barja, 1993), the great majority of investigations concerns AOS toxicity. It is postulated that the delicate balance between production and catabolism of AOS (the balance between prooxidant factors and antioxidants) is held. If this balance is upset, either by overproduction of AOS and/or due to reduced effectiveness of the defence mechanism, excess concentrations of AOS result, leading to the tissue damage (reviewed recently by Barja, 1993). Superoxide dismutase, catalase and glutathione peroxidase (non-heme peroxidase) are the main antioxidant enzymes in cells. Contrary to the superoxide anion and hydrogen peroxide, there is no specific defence mechanism against hydroxyl radicals produced in the cells.

The role of heme peroxidases as defence enzymes is discussible. They catalyze the oxidation of variety of substrates by peroxides resulting in production of substrate free radicals:

$$\text{Peroxidase} + H_2O_2 \rightarrow \text{Compound I} \quad (1)$$
$$\text{Compound I} + RH \rightarrow \text{Compound II} + R^{\cdot} \quad (2)$$
$$\text{Compound II} + RH \rightarrow \text{Peroxidase} + R^{\cdot}, \quad (3)$$

where RH is a substrate, and $R^{\cdot}$ is a substrate radical.

The substrate radicals may dimerize, disproportionate or may be scavenged by molecular oxygen yielding peroxy radicals or superoxide anion (Metodiewa & Dunford, 1993). On the other hand, peroxidases and their compounds I are known to scavenge superoxide anion.

It has been suggested that $OH^{\cdot}$ radicals induce oxidative cleavage of the porphyrin ring structure of the heme moiety in lactoperoxidase leading to the liberation of the iron ions (Jenzer, Kohler & Borger, 1987).

In this work, the reactions of heme peroxidases, both plant (horseradish peroxidase-HRP) and animal (lactoperoxidase-LPO) with hydroxyl radicals beeing in excess towards enzymes have been studied. The reactions of compound II of peroxidases with superoxide anion have been also investigated. Oxygen radicals ($OH^{\cdot}$ and $O_2^{\cdot-}$) have been generated by means of pulse radiolysis.

EXPERIMENTAL

HRP (type VI) with RZ (A_{403} /A_{280}) of 3.0 and LPO with RZ (A_{412}/A_{280}) of 0.84 were obtained from Sigma. All experiments were carried out at ambient temperature and at pH 7.0 (1 mM phosphate buffer). Pulse radiolysis experiments were performed with the use of linear accelerator at the Institute of Applied Radiation Chemistry, Techni-

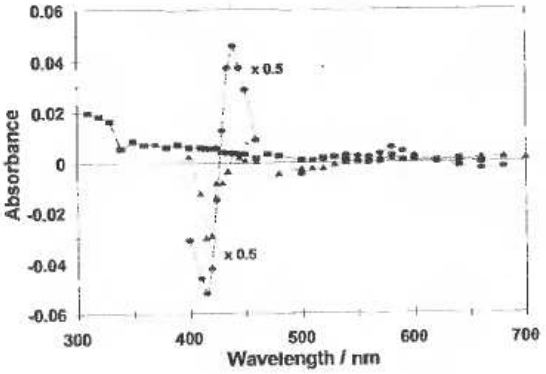

Fig. 1. Difference absorption spectra taken 10 μs (circles), 2 ms (squares) and 100 ms (triangles) after 1 μs pulse (150 Gy) to N_2O-saturated 2.2 μM LPO aqueous solution.

cal University of Łódź. The accelerator and the detection system have been described elsewhere (Karolczak, Hodyr, Łubis & Kros, 1986; Karolczak, Hodyr & Połowiński, 1992). Reactions of $OH^{\cdot}$ radicals with peroxidases were studied in N_2O-saturated aqueous solutions of the enzymes, where all e^-_{aq} were converted to $OH^{\cdot}$. Reactions of $O_2^{\cdot-}$ radical ions with peroxidases were observed in O_2-saturated aqueous solutions of the enzymes, in the presence of 0.1 M sodium formate to scavenge $OH^{\cdot}$ radicals. In both cases H_2O_2 was produced in the pulse with the yield $G(H_2O_2)$ = 0.07 μM J^{-1}. Pulses of 5 ns or 1 μs delivering doses of 10 and 150 Gy (J kg^{-1}), respectively, were applied.

RESULTS AND DISCUSSION

The reactions of hydroxyl radicals with peroxidases

Protein sites of known reactivity towards $OH^{\cdot}$ are aromatic and heterocyclic peptide residues (addition) and ubiquitous CH groups (H-atom abstraction). In our earlier study we have found that under conditions, where an excess of peroxidase was used, the oxidizing radical $OH^{\cdot}$ is involved, as a precursor, in reduction of the heme iron. The process takes part in hundreds of miliseconds after $OH^{\cdot}$ generation (Gębicka & Gębicki, 1992). The explanation for this unexpected heme reduction observed also for other hemoproteins is, that the initial radical, formed on the protein molecule, reacts subsequently to release an electron which then migrates to the heme (Klapper & Faraggi, 1979). Our spectral data (Gębicka & Gębicki, 1992) indicate that roughly 15% of the hydroxyl radicals are involved in the reduction of the investigated enzymes.

Absorption changes observed in N_2O-saturated, pulse irradiated enzyme solution, under conditions, where hydroxyl radicals were generated in great excess over enzyme (dose 150 Gy, $[OH^{\cdot}]$=80 μM) are shown in Fig 1. Spectrum taken 10 μs after the pulse rising towards UV is connected with primary products of $OH^{\cdot}$ attack on protein molecule. We do not observe any bleaching of the Söret band for both enzymes, what means that $OH^{\cdot}$ radicals do not react directly with heme. Absorption spectra taken in milliseconds after the pulse are characteristic for HRP and LPO compounds I (data shown only for LPO). Under our experimental conditions 10.5 μM H_2O_2 was produced in the pulse and some amounts of H_2O_2 was formed additionally from $OH^{\cdot}$ recombination ($k_{OH^{\cdot}+OH^{\cdot}} = 5.5 \cdot 10^9$ M^{-1} s^{-1}, Buxton, Greenstock, Helman & Ross, 1988). It is known, that when H_2O_2 is in excess, compounds I of HRP and LPO convert to the respective compounds II. However, the rate of the formation of compound II in our pulse-radiolysis experiments exceeded by ca one order of magnitude the rate of spontaneous compound I/compound II conversion observed by us at comparable enzyme/H_2O_2 concentration in stopped-flow experiments (our unpublished results). Similar observations has been done earlier with HRP exposed to e^-_{aq}/H_2O_2 (Gębicka & Gębicki, 1991). The formation of compound II involves one-electron reduction of the porphyrin π-cation radical (compound I) leaving heme iron with the ferryl structure. Electrons involved in the process may very well be transferred from radicals that are formed by the interaction of $OH^{\cdot}$ with the protein moiety. As the formation of compound I is relatively fast ($k \approx 2 \cdot 10^7$ M^{-1} s^{-1}, Dunford & Stillman, 1976), the reduction of ferric iron through electron transfer from protein radicals

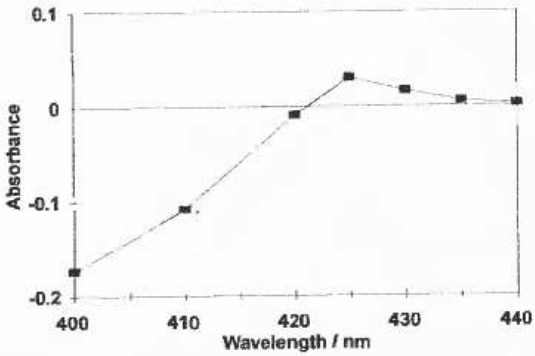

Fig 2. Difference absorption spectrum taken 100 ms after 5 ns pulse (10 Gy) to O_2-saturated 36 μM HRP compound II aqueous solution.

seems to be negligible under conditions described above.

The reactions of superoxide radicals

It is known that hemoenzymes (catalase, peroxidases) react with superoxide radicals to form catalytically inactive compound III (Kono & Fridovich, 1982, Metodiewa & Dunford, 1989). Superoxide anion is also known to reduce compound I to compound II in catalase (Gębicka, Metodiewa & Gębicki, 1989), HRP (Bielski, Comstock, Haber & Phillip, 1974) and LPO (Gębicka & Gębicki, 1993). There is a controversy about the possibility of the reaction of compound II with $O_2^{\cdot-}$. Such reaction was observed for myeloperoxidase (Kettle, & Winterbourn, 1988), horseradish peroxidase (Metodiewa & Dunford, 1992) and very recently for *Aspergillus niger* catalase (Lardinois & Rouxhet, 1994). In these cases the native enzyme was suggested to be the product of this reaction. On the other hand Bielski *et al.* (1974) and Kobayashi *et al.* (Kobayashi, Hayashi & Swallow, 1990) did not see any reaction.

We have studied reactions of preformed compound II of HRP and LPO with $O_2^{\cdot-}$. Compound II of HRP was produced by mixing O_2-saturated enzyme solution with tenfold excess of H_2O_2 and remained stable over ca 15 min. Compound II of LPO was produced by mixing O_2-saturated enzyme solution with 2.5-fold excess of H_2O_2 and remained stable over ca 10 min. Pulse-irradiation was carried out under conditions, of compound II excess over $O_2^{\cdot-}$. Absorption spectrum taken 100 ms after the pulse-irradiation of O_2-saturated solution of HRP compound II is shown in Fig. 2. A strong bleaching of Söret band indicates that the interaction of $O_2^{\cdot-}$ with the heme group leads to the formation of a new derivative, stable over our longest observation time, i.e. up to 7 s. The nature of it has not been established yet. Similar behaviour was observed for LPO compound II. The discrepancies, concerning the reaction of HRP compound II with $O_2^{\cdot-}$, between different groups (Bielski *et al.*, 1974; Kobayashi *et al.*, 1990: Metodiewa & Dunford, 1992 and this work) are not clear at present. The stopped-flow experiments with superoxide generated either chemically or enzymatically are planned to shed more light on this problem.

Acknowledgement

This work was partly supported by grant KBN 0142/P2/94/06

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Current Topics in Biophysics, vol. 18(2), 184-188.

ALTERATIONS IN THE SUSCEPTIBILITY TO TRYPSINOLYSIS OF PROTEINS CHLORINATED AND/OR EXPOSED TO SIN-1

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(Accepted 28 December 1994)

The respiratory burst of neutrophilic granulocytes results in production of highly reactive species, among which the HOCl/ClO⁻ system is formed. This system may react with proteins and the modifications due to chlorination change the protein vulnerability to enzymatic proteolysis. The aim of this study was to test if the nitric oxide free radical ($NO^{\cdot}$) or its derivatives produced by activated phagocytes and showing cytotoxic properties, may act in a similar way and modify proteins which afterwards are more efficiently digested by proteolytic enzymes. Bovine serum albumin (BSA) and ovalbumin (OVA) were chlorinated with a concentration of ClO⁻ causing the highest enhancement of proteolytic degradation and/or exposed in aerobic conditions to SIN-1 (3-morpholino-sydnonimine, a $NO^{\cdot}$ and $O_2^{\cdot-}$ groups donor) in the following concentrations: 10, 100, and 1000 nmol/ml of the protein solution. Proteolysis of modified proteins was traced as an increase of free amino groups measured by the TNBS (2,4,6-trinitro-benzenesulfonic acid) method. It was found that free radicals released from SIN-1 or their derivatives have only a slight effect on the rate of BSA digestion, both native and previously chlorinated. (Digestion of chlorinated BSA was 1.7 times faster than that of the native protein). In contrast to BSA, OVA treated with SIN-1 in concentration of 100 nmol/ml underwent degradation 1.7 times faster than its native form. When the concentration of SIN-1 was 1000 nmol/ml, the trypsinolysis was 3.2 times faster, while OVA modified with 10 nmol/ml SIN-1 was digested slower (0.7 times) after its modification than the native form. OVA exposed to SIN-1 after pretreatment with ClO⁻ was a better substrate for trypsin than the native protein; the digestion was 4 – 5.9 times faster for all concentrations of SIN-1. (Digestion of chlorinated OVA was 3.6 times faster than that of the native protein).

INTRODUCTION

Free radicals arise during various biological processes including electron transport in mitochondria and some metal-catalysed oxidations. Increased formation of free radicals has been implicated in many human diseases (Änggard 1994; Ferrante, Kowanko & Bates, 1992; Halliwell, Gutteridge, Cross, 1992).

During inflammation, activation of phagocytes results in production of primary and secondary products which are toxic to ingested microorganisms and extracellular targets but also harmful to neighbouring tissues. Due to NADPH oxidase action the superoxide anion radical $O_2^{\cdot-}$ is formed. Dismutation of $O_2^{\cdot-}$ results in the formation of H_2O_2, which can react with a peroxidase and chloride to form hypochloride ions ClO^-. Hypochlorides are oxidizing as well as chlorinating species. Tissue injury resulting from an acute inflammatory response is linked to the presence of proteolytic enzymes and toxic oxygen products released from activated neutrophils, monocytes and macrophages (Drożdż & Naskalski, 1988; Ferrante *et al.*, 1992; Klebanoff, 1993; Zgliczyński & Stelmaszyńska, 1988).

Most proteins are susceptible to oxidative modifications and the modified proteins are rapidly and selectively degraded by proteolytic enzymes due to the alterations in their primary, secondary, and tertiary structure (Davies 1987; Davies & Delsignore, 1987; Davies, Delsignore & Lin, 1987a; Davies, Lin & Pacifici, 1987b).

Generation of nitric oxide ($NO^{\cdot}$) by arginine-dependent pathways may injure tissue due to the toxic effects of $NO^{\cdot}$ or its derivatives such as the formation of $HO^{\cdot}$ as a result of $NO^{\cdot}$ and $O_2^{\cdot-}$ interaction (Beckman, Beckman, Chen, Marshall & Freeman, 1990; Blough & Zafiriou, 1985; Kolb & Kolb-Bachofen, 1992; Mulligan, Hevel, Marletta & Ward, 1991). It is therefore possible that the

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The laboratory work was completed in the Department of Immunology, Collegium Medicum, Jagiellonian University, Kraków.
We would like to express our special thanks to Prof. W. Ptak.

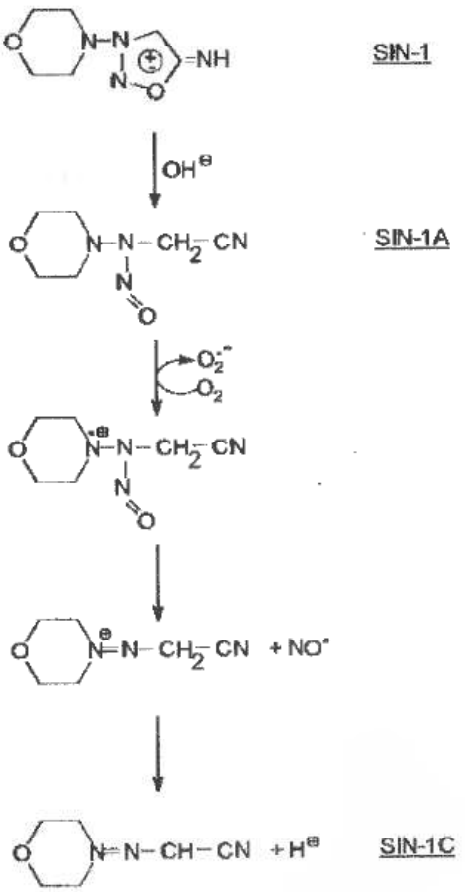

Fig. 1. $NO^{\cdot}$ and $O_2^{\cdot-}$ formation during SIN-1 decomposition (Bohn & Schoenafinger, 1989; Jessup *et al.*, 1992; Noack & Feelisch, 1989).

synthesis of $NO^{\cdot}$ alone or $NO^{\cdot}$ plus $O_2^{\cdot-}$ by phagocytes could lead to the formation of oxidizing species that contribute to the modifications of proteins.

The objective of the present study was to investigate the alterations in proteolytic susceptibility of proteins exposed to hypochloride and/or reactive nitrogen intermediates formed during the decomposition of SIN-1.

MATERIALS AND METHODS

Bovine serum albumin, egg white albumin, and phenylmethane-sulfonyl fluoride (PMSF) were obtained from Sigma Chemical Co. (USA). Trypsin 1:250 was from Difco Laboratories (USA). Sodium hypochlorite solution was purchased from BDH (UK). TNBS was obtained from Eastman Kodak Co. (USA). SIN-1 was a generous gift from GEA, Denmark. The other reagents were of analytical grade from P.O.Ch. (Poland).

Table 1. The rate of trypsinolysis of native and modified proteins.

Modifying agent		Rate of trypsinolysis	
ClO^-	SIN-1	BSA	OVA
[10 μmol/ml]	[μmol/ml]	%	%
–	–	100	100
–	10	110	70
–	100	110	170
–	1000	110	320
+	–	170	360
+	10	170	590
+	100	160	450
+	1000	110	410

Spectrophotometric measurements were carried out with a UV-VIS spectrophotometer Gilford-Response II.

NaOCl stock solution was standardized iodometrically as described by Stelmaszyńska and Zgliczyński (1978).

Prior to exposure to the modifying agents, BSA and OVA were dialysed overnight against 0.05M phosphate buffer, pH 7.6 to get rid of the sulfate remainder.

Protein chlorination

Protein chlorination was performed according to Olszowska *et al.* (Olszowska, Olszowski, Zliczyński & Stelmaszyńska, 1989). Briefly, BSA and OVA at a concentration of 2 mg per ml of 0.05 M phosphate buffer, pH 7.6, were dialysed against outer 0.05 M buffer containing NaOCl at a concentration of 10 μmol per ml of the protein solution. Dialysis lasted for 72 hr at 4°C. In order to reduce the excess of ClO^- and N-chlororesidues, after chlorination the samples were treated with thiosulfate at the thiosulfate-chlorinating agent molar ratio 1:1 and subsequently dialysed against phosphate buffer overnight.

Exposure of proteins to SIN-1

The proteins were incubated overnight at 20°C in aerobic 0.05 M phosphate buffer, pH 7.6, containing SIN-1 at the concentrations of 10, 100, and 1000 nmol per ml of the protein solution.

SIN-1C (N-morpholinoiminoacetonitrile) solution

1000 nmol/ml SIN-1C solution was prepared by dissolving the equimolar amount of SIN-1 in 0.05 M phosphate buffer, pH 7.6, and incubating it in 20°C overnight.

Quantitation of proteolysis

Trypsinolysis of unmodified and modified proteins was traced as an increase of free amino

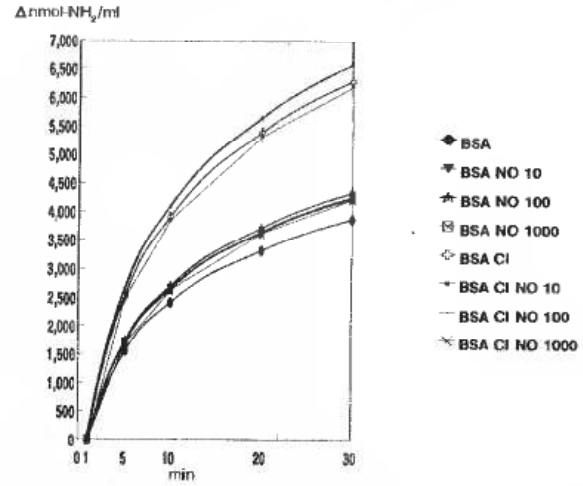

Fig. 2. Effect of albumin chlorination and/or exposition to SIN-1 on the susceptibility of this protein to trypsinolysis.

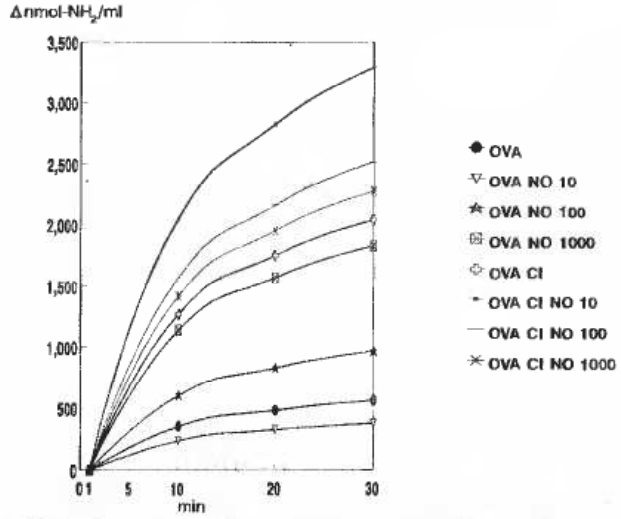

Fig. 3 Effect of ovalbumin chlorination and or exposition to SIN-1 on the susceptibility of this protein to trypsinolysis.

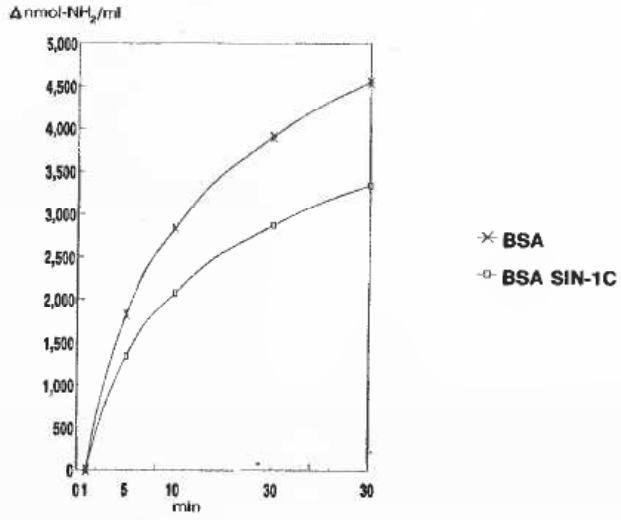

Fig. 4. Effect of SIN-1C on the rate of albumin hydrolysis by trypsin.

groups measured by the TNBS method (Spadaro, Camargo & Greene, 1979) with modification of Olszowska *et al.* (1989).

RESULTS

The extent of chlorination is crucial for the protein predisposition to proteolysis. With the concentration of 10 µmol NaOCl per ml of the protein solution the effect obtained is maximal (Olszowska *et al.*, 1989), so this concentration was applied in our investigation.

Trypsinolysis of chlorinated BSA was 1.7 times faster than the digestion of the native form; OVA showed 3.6 times acceleration in the proteolytic degradation (Fig. 1, 2).

The ring-opening reaction of SIN-1 to SIN-1A (N-morpholino-N-nitrosoamino-acetonitrile) is pH-dependent. During the aerobic decomposition of SIN-1A, free radicals ($NO^{\cdot}$ and $O_2^{\cdot-}$) and SIN-1C are formed (Fig. 1).

BSA and OVA incubated with SIN-1 under aerobic conditions show alterations in their susceptibility to trypsinolysis (Fig. 2, 3, Tab. 1).

To eliminate the possible influence of SIN-1C on the action of trypsin, the native BSA was incubated with SIN-1C and trypsin. The rate of digestion reached 0.8 of that when incubated without SIN-1C (Fig. 4).

DISCUSSION

Proteins exposed to oxygen free radicals undergo alterations to primary, secondary, and tertiary structure. Although the mechanism by which proteolytic enzymes recognize oxidatively modified proteins are not well understood, the declining proteolytic susceptibility may be explained by the increase in protein cross-linking, whereas enhanced proteolytic degradation may be a result of denaturation or fragmentation (Davies 1987; Davies & Delsignore, 1987; Davies *et al.*, 1987a, b; Grant, Jessup & Dean, 1992, 1993).

$NO^{\cdot}$ is a weak reducing agent. It was reported by Jessup *et al.* (1992), that only $NO^{\cdot}$ together with $O_2^{\cdot-}$, but not $NO^{\cdot}$ alone, even under aerobic conditions, can efficiently oxidize LDL antioxidants and lipids. As phagocytes can produce both $O_2^{\cdot-}$ and $NO^{\cdot}$, we tested the effect of SIN-1 on BSA and OVA. SIN-1 decomposes to SIN-1C with concominant production of $NO^{\cdot}$ and $O_2^{\cdot-}$ (Fig. 1). $NO^{\cdot}$ rapidly reacts with $O_2^{\cdot-}$ to form peroxynitrite anion ($ONOO^-$) in high yield. Once protonated, it decays rapidly (Beckman *et al.*, 1990):

$$O_2^{\cdot-} + NO^{\cdot} \rightarrow ONOO^- + H^+ \leftrightarrow ONOOH \rightarrow HO^{\cdot} + NO_2^{\cdot} \rightarrow NO_3^- + H^+$$

The products formed are potent oxidants that mediate oxidation of both nonprotein and protein sulfhydryls (Radi, Beckman, Bush & Freedman, 1991), hydroxylation and polymerization of phenolic rings (Grant *et al.*, 1992), oxidation of lipids and LDL antioxidants (Jessup & Dean, 1993; Jessup, Mohr, Gieseg, Dean & Stocker, 1992), inhibition of various enzymes with iron – sulfur centres (Davies *et al.*, 1987b; Lancaster & Hibbs, 1990), hem enzymes (Davies *et al.*, 1987b), DNA damage (Davies *et al.*, 1987b), and form stable chelates with intracellular stores of iron and other transition metals (Jessup & Dean, 1993).

Sulfhydryl oxidation is a key mechanism of free radical – mediated cytotoxicity at the molecular level (Radi *et al.*, 1991). The radical – damaged protein is more rapidly endocytosed (Davies & Delsignore, 1987) and degraded (Davies & Delsignore, 1987; Olszowska *et al.*, 1989).

SIN-1 produces equimolar amount of $NO^{\cdot}$; the concentration of 100 nmol/ml is achieved in cultures of IFN-γ activated macrophages (measured as NO_2^- concentration), (Stuehr & Nathan, 1989). The *in vivo* local $NO^{\cdot}$ concentration is difficult to estimate, so in our experiments we used a 10-times higher concentration of SIN-1 as well.

Our results show that the vulnerability of the native BSA remains almost completely unchanged after exposure to all applied concentrations of SIN-1. Although chlorination enhances the proteolytic degradation of BSA, subsequent incubation with SIN-1 did not cause much alterations. Davies (1987) presented a possible explanation of such a phenomenon. Globin is a denaturated protein, and casein has little, if any, secondary or tertiary structure. Since untreated globin and casein were both excellent proteolytic substrates, they may have reached their maximal rate of degradation before the exposure to $HO^{\cdot}$.

It is highly possible that in our experiments trypsin attained the maximal activity with BSA chlorinated under conditions described above and that subsequent modifications by highly active species could only drop down the efficiency of proteolysis.

OVA, in comparison to BSA, showed twice higher vulnerability to digestion when exposed to the same concentration of chlorinating agent, moreover, incubation with SIN-1 of both, native

and chlorinated, forms resulted in significant acceleration of proteolytic degradation.

An explanation of these differences needs further investigation.

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Current Topics in Biophysics, vol. 18(2), 189-194

ACCELERATION OF TRANSFORMATION OF THIYL RADICALS TO DISULFIDES BY METHEMOGLOBIN. ANTIOXIDANT FUNCTION OF METHEMOGLOBIN

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(Accepted 28 December 1994)

Glutathione and cysteine oxidation to disulfidic forms under the influence of oxygen free radicals, generated by ultrasound was observed. MetHb catalysed dimerization of GS˙ thiyl radicals to disulfides and was reduced to the ferro form. It is assumed, that GS thiyl radicals react with glutathione GS⁻ to form intermediate ion-radical species of GSSG glutathione, which reduces metHb to the ferro form. Thiyl radicals do not oxidize oxyHb or carboxyHb to metHb. MetHb increases the resistance of glutathione to ultrasound.

INTRODUCTION

It is well known that glutathione and other thiols provide protective function by decreasing oxygenous and organic free radical damage in biological systems (Willson, 1985; Keniya, Lukash & Guskou, 1993). Free radicals are generated during xenobiotic metabolism by several enzymes (Slater, 1972), in the course of hydroperoxide transformations initiated by transition metal ions, in biological objects exposed to ionizing radiation (Mason, 1982), during oxyhemoglobin oxidation (Wallace, Maxwell & Canghey, 1974), under the ultrasound action in water solutions (Makino, Massoba & Riesz, 1983).

Glutathione is one of the most important intracellular thiols; its concentrations reaches up to $2 \cdot 10^{-3}$ M in erythrocytes and 10^{-2} M in other cells (Pennel, 1964).

The protective effect of glutathione is due to its reactions with oxygen and organic free radicals via hydrogen atom abstraction from thiol molecule (Schoneich, Bonifacic & Asmus, 1989a). Thiyl radicals being formed have less detrimental effect comparing with radicals, with unpaired electron localized at the carbon atom. Moreover, thiyl radicals are eliminated from reaction medium very effectively because of glutathione disulfide formation (Ross, Norbeck & Moldeus, 1985).

However, thiyl radicals can cause membrane damage due to interaction with unsaturated fatty acids resiues of membrane phospholipids such as arachidonic, linolenic, and linoleic residues (Aqino, Dunster & Wilson, 1989). Perhaps, interaction of thiyl radicals with vitamin A or ascorbic acid with high rate constant is the mechanism decreasing RS-radical antioxidant and antiradical activities (Keniya *et al.*, 1993; Aqino *et al.*, 1989).

It is shown in a number of reports, that methemoglobin has antioxidant function and oxidizes many organic free radicals, for example, of aliphatic alcohols or NAD (Stepuro, Ignatenko & Oparin, 1991; Stepuro, Oparin & Kusnetsova, 1992; Stepuro & Konovalova, 1992).

It is shown in this work that methemoglobin cause thiyl radicals dimerization in to disulfides, being itself reduced to the ferro form. Oxyhemoglobin or carboxyhemoglobin do not interact with thiyl radicals.

MATERIALS AND METHODS

The GSH concentration was determined spectrophotometrically using 5,5-dithiobis-(2-nitrobenzoic acid) by measuring absorption at 412 nm (Torchynsky, 1972). OxyHb was obtained by the technique described previously (Benesch, Benesch, Renthol & Macda, 1972). CarboxyHb was obtained by passing of carbonic oxide formed by reaction of sulphuric acid with formic acid through oxyHb solution. Concentrations of the ferro and ferri forms of hemoglobin were determined spectrophotometrically using molar extinction coefficient of ε_{542} = 14370 $M^{-1} \cdot cm^{-1}$ for oxyHb, ε_{630} = 3700 $M^{-1} \cdot cm^{-1}$ for metHb and ε_{539} = 14360 $M^{-1} \cdot cm^{-1}$ for carboxyHb (Kampen & Zijlstra, 1983). Hb ferryl form was registered by sulfhemoglobin formation after incubation with Na_2S (Kampen & Zijlstra, 1983). Oxygen free

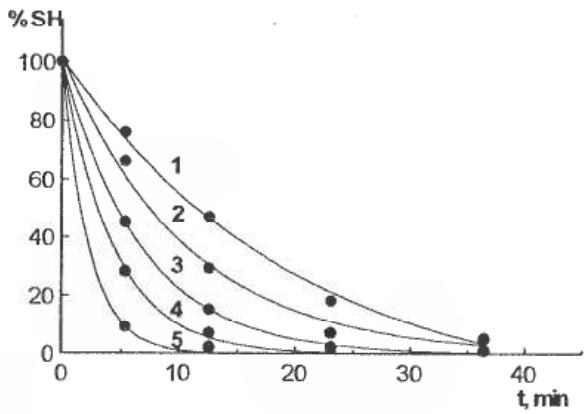

Fig. 1. Oxidation kinetics of aqueous and aqueous-alcoholic solutions of glutathione and cysteine under the influence of free radicals. Aqueous glutathione solution under nitrogen atmosphere (1). Aqueous-alcoholic solution of glutathione: (2), aqueous glutathione solution: (3), aqueous-alcoholic solution of cysteine: (4), aqueous cysteine solution in air atmosphere: (5). The concentration of glutathione, cysteine and ethanol in the solutions were $5 \cdot 10^{-4}$ M, 10^{-4} M and 1 M, respectively. The solution pH was always 6.8.

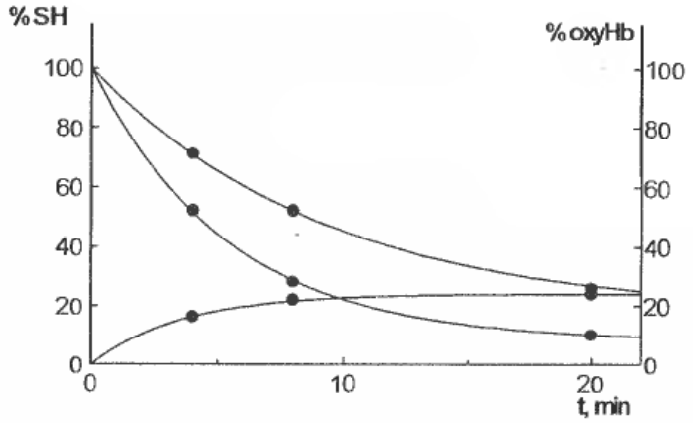

Fig. 2. Oxidation kinetics of glutathione (1) and its mixture with metHb (2); metHb reduction kinetics in the presence of glutathione (3). The concentrations of glutathione and metHb were 10^{-3} M and $3 \cdot 10^{-5}$ M, respectively. pH of aqueous solutions was 6.8.

radicals generation was performed by ultrasonic irradiation of aqueous solutions of glutathione and hemoglobin. The absorption spectra in the UV and visible range were recorded in a "Specord M-40" spectropotometer (Germany).

RESULTS

Glutathione was oxidized almost completely to the disulfide form on exposure to hydroxyl radicals, superoxide anions as well as HO_2, H_2O_2 and nitrogen oxides generated by ultrasound in water solutions and in air (Fig. 1). Sulfhydryl groups formation was observed after glutathione oxidation products had been treated by $NaBH_4$. S-nitrosoglutathione formation was insignificant for glutathione concentrations $1 \cdot 10^{-4} - 5 \cdot 10^{-4}$ M, and futher deep oxidation products, for example GSO_3H, were produced only after ultrasound exposure of the disulfide form.

Glutathione oxidation rate was decreased in anaerobic atmosphere (after blowing of N_2 or CO) in consequence of lowering of $O_2^{\cdot}$, $HO_2^{\cdot}$ and H_2O_2 generation. If metHb is added to glutathione solution, glutathione oxidation rate increases and a small fraction of metHb is reduced to deoxyHb which is oxygenated in air atmosphere (Fig. 2, curve 2).

In solution exposed to ultrasound the proportion of hemoglobin ferro and ferri forms reaches a stationary value and the rate of glutathione oxidation to the disulfide form increases. As shown in Fig. 2, the initial methemoglobin concentration was 10^{-5} M and the glutathione concentration was 10^{-4} M. This low metHb concentration as compared with GSH catalyzed glutathione oxidation. GSH oxidation rate was decreased in the presence of oxyHb and the rise of glutathione oxidation rate was observed only after the transformation of a considerable fraction of oxyhemoglobin to metHb (Fig. 3). Actually, when the concentration of oxyHb was higher than that of glutathione, oxyHb was oxidized to metHb. On the contrary hemoglobin oxidation was insignificant in the presence of a high molar excess of GSH in solution and in the absence of oxygen (CO or N_2 atmosphere) (Fig. 4). Probably, these data suggest the participation of active oxygen forms in ferro hemoglobin

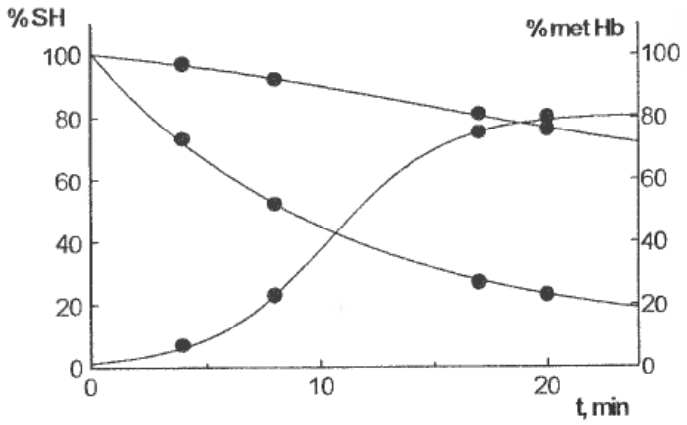

Fig. 3. Oxidation kinetics of glutathione, glutathione in the presence of oxyHb and oxyHb in the presence of glutathione in ultrasonic field. The decrease of sulfhydryl groups of glutathione non-mixed (1) and mixed (2) with oxyHb and oxyHb oxidation to metHb in the presence of glutathione (3). The concentrations of glutathione and oxyHb were 10^{-3} M and $3 \cdot 10^{-5}$ M, respectively. pH of aqueous solutions was 6.8.

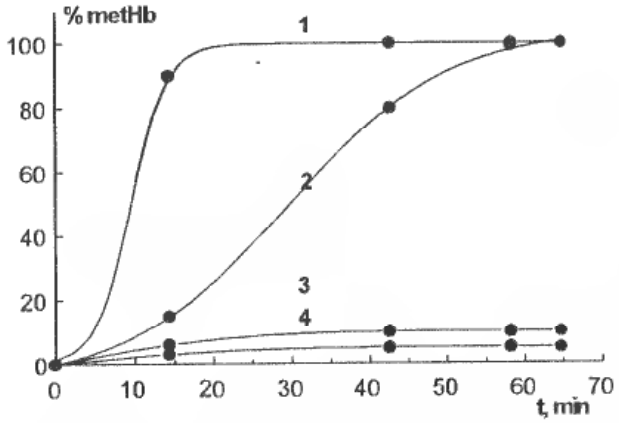

Fig. 4. Oxidation kinetics of oxyHb to metHb in ultrasonic field in air atmosphere (1), in carbonic oxide atmosphere (2), and that of oxyHb in carbonic oxide atmosphere in the presence of glutathione (3, 4) at the concentrations of 10^{-3} and $5 \cdot 10^{-3}$ M, respectively. OxyHb concentration was $3 \cdot 10^{-5}$ M; the solution pH was 7.0.

oxidation (both HbO_2 and HbCO) (Fig. 4, curve 1 and curve 2).

Thiyl radicals generated by ultrasonic treatment of water-alcohol solutions containing thiol compounds did not oxidize oxyHb. MetHb degradation rate decreased rapidly in the presence of the glutathione (Fig.5).

DISCUSSION

The interaction of $^{\cdot}OH$, $HO_2^{\cdot}$, $O_2^{\cdot-}$ oxygen free radicals, and of hydrogen peroxide generated in ultrasonic field with thiol compounds leads to the formation of thiyl radicals:

$$GSH + HO^{\cdot} \rightarrow GS^{\cdot} + H_2O$$

$$GSH + O_2^{\cdot-} \xrightarrow{H^+} GS^{\cdot} + H_2O_2$$

then, thiyl radicals dimerize to disulfides:

$$GS^{\cdot} + GS^{\cdot} \xrightarrow{k} GSSG$$

where $k = 3.5 \cdot 10^9$ $M^{-1} \cdot s^{-1}$ (Hoffman & Hayan, 1972).

The rate constant for thiyl radicals interaction with OH is by several orders of magnitude higher than those for their interaction with O_2 and $HO_2^{\cdot}$. This is the reason of relatively small dependence of oxidation rate of GSH or cysteine on the presence of oxygen atmosphere in ultrasonic field (Fig. 1). In a first approximation we can consider that hydroxyl radicals make the main contribution in GSH oxidation.

MetHb catalyzes thiyl radicals dimerization into disulfide forms with reduction to deoxyHb, which in the presence of gas ligands is convert into appropriate ligandic ferro form such as oxyHb or carboxyHb (Fig. 3). Thiyl radicals interact with GS^- and glutathione ion-radical is formed:

$$GS^{\cdot} + GS^- \rightarrow G\dot{S}\bar{S}G$$

with $k = 6.6 \cdot 10^8$ $M^{-1} \cdot s^{-1}$ (Ross *et al.*, 1985).

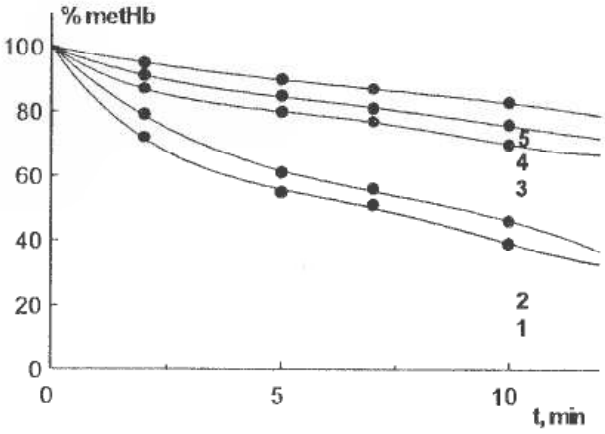

Fig. 5. Degradation kinetics of metHb and metHb in the presence of glutathione. Hemine content decrease in aqueous solution of metHb in the absence (1) and in the presence (4) of glutathione and in aqueous-alcoholic solution in the presence of glutathione (5). Fluorescence intensity decrease of metHb tryptophane in aqueous solutions in the absence (2) and in the presence (3) of glutathione. Glutathione and metHb concentrations were 10^{-3} M and $2 \cdot 10^{-5}$ M, respectively. The solution pH was 7.0. Absorption at 406 nm (at the Soret band) and tryptophane fluorescence intensity at 350 nm with excitation at 296 nm were measured. Initial optical density of metHb and the initial tryptophan fluorescence intensity were considered as 100%.

Probably that intermediate product is an effective donor of electrons. Therefore, interacting with metHb it reduces the latter to the ferro form:

$$Hb(III) + G\,S\,\overline{S}G \rightarrow Hb(II) + GSSG\,.$$

In the absence of glutathione, metHb rapidly degrades in the ultrasonic field with the formation of the ferryl form, oxidation of cysteine and aromatic amino acid residues, oxidation of porphyrine IX ring and Fe (III) release (Fig. 5).

The existence of the ferryl form is proved by sulfhemoglobin formation after Na_2S had been added in sonicated metHb solutions.

MetHb molecule contains many imidazole, thyrosine and cysteine residues which are effective traps of hydroxyl radicals and of other active oxygen forms. Amino acid side residues cation-radicals formed are probably inactivated by $GS\,\overline{SG}$:

$$Hb^{+}(III) + GS\,GS \rightarrow Hb(III) + GSSG\,.$$

This leads to metHb protection against further damages associated with irreversible denaturation of the protein globule. Therefore, GSSG participation in the reaction of metHb or its free radical forms increases disulfide formation of GSSG, causes metHb reduction to deoxy form, and protects the protein globule from damage. Due to the high reaction rate constants of oxygen free radicals for deoxyHb oxygenation competitive reactions will proceed with accumulation of some quantity of oxyHb (Fig. 3). As a result, stationary concentrations will establish where metHb and oxyHb levels are at equilibrium, and GSH oxidation rate to GSSG is higher than when metHb is absent (Fig. 3).

Under the influence of oxygen free radicals generated by ultrasound on HbO_2 or HbCO, their rapid oxidation in metHb without protein globule damage was observed (Stepuro *et al.*, 1992).

$$HbO_2 + HO^{\cdot} \rightarrow Hb(III) + HO^{-} + O_2$$

$$HbO_2 + HO_2^{\cdot} \rightarrow Hb(III) + O_2 + H_2O_2$$

Hydrogen peroxide interacts with metHb and ferryl form is formed:

$$Hb(III) + H_2O_2 \rightarrow Hb(IV) - OH + HO^{-}\,.$$

Ferryl form interacts with HbO_2 and metHb is formed:

$$Hb(IV) - OH + HbO_2 \rightarrow 2metHb + HO^{-} + O_2^{\cdot}\,.$$

Consequently, metHb can be considered as the main product of Hb oxidation. Practically full protection of glutathione or cysteine from oxidation to disulfides by oxygen free radicals was observed in cases when the excess of HbO_2 was sufficiently high. Thiol oxidation occurred only after increase of Hb(III) content (Fig. 2).

The oxyHb oxidation in oxygen atmosphere in the presence of GSH is probably accompanied by formation of $O_2^{\cdot}$ radicals in the disulfide anion-radical conversions process:

$$G\dot{S}\,\overline{S}G + O_2 \rightarrow O_2^{\cdot} + GSSG\,.$$

Superoxide anion dismutates to O_2 and hydrogen peroxide; the latter is known to oxidize oxyHb to metHb. Besides, hydrogen peroxide forms in cavitation bubbles, diffuses in solution and interacts with Hb.

In the presence of aliphatic alcohols, which are effective traps of $^{\cdot}OH$ and $H^{\cdot}$ radicals, the transformation of $^{\cdot}OH$ and $H^{\cdot}$ in to organic free radicals occurs:

$$CH_3CH_2OH \xrightarrow{H} CH_3\,CH\,OH + H_2$$
$$CH_3CH_2OH \xrightarrow{HO} CH_3\,CH\,OH + H_2O\,.$$

Alcohols have high vapour pressure, and in cavitation bubbles a decrease of free radical reactions with oxygen and of the reactions of free radical recombination is observed:

$$H^{\cdot} + O_2 \rightarrow HO_2^{\cdot};\quad H^{\cdot} + H^{\cdot} \rightarrow H_2;$$
$$H^{\cdot} + HO^{\cdot} \rightarrow H_2O;$$
$$HO^{\cdot} + HO^{\cdot} \rightarrow H_2O_2;\ HO_2^{\cdot} + HO_2^{\cdot} \rightarrow H_2O_2 + O_2$$

Hydrogen peroxide in ultrasonic field did not form when ethanol concentrations were higher than 10^{-1} M. This proves the almost complete interception of $^{\cdot}H$ and $^{\cdot}OH$ radicals by ethanol vapour in cavitation bubbles.

Alcohol free radicals oxidize thiols and thiyl radicals are formed:

$$CH_3\,CH\,OH + GSH \underset{k_2}{\overset{k_1}{\rightleftarrows}} G\,S + CH_3CH_2OH\cdot$$

where $k_1 = 10^8\ M^{-1}s^{-1}$, $k_2 = 1.2\cdot10^4\ M^{-1}s^{-1}$ (Schoneich *et al.*, 1989b).

As the rate constant values k_1 and k_2 show, in water-alcohol solution containing thiols almost complete transformation of alcohols radicals in to thiyl radicals can be obtained. Since transformations of oxyHb, HbCO to metHb were not observed in the presence of alcohols and thiols, then thiyl radicals most probably do not oxidize ligandic ferro forms of Hb.

Summarizing the results, we can conclude that metHb catalyses thiyl radicals dimerization. We assume that interaction of $GS\dot{S}G^-$ anion-radical with metHb occurs. We suppose that the same acceleration mechanism of the thiyl radicals can operate in living cells. As it is known GSH level can achieve 10^{-2} M in liver cell, and in erythrocytes it is $2\cdot10^{-3}$ M. Glutathione is one of the most powerful low molecular antioxidant (Keniya *et al.*, 1993; Ross *et al.*, 1985) and it interacts with free radicals with formation of thiyl radicals. The thiyl radicals are removed by formation of GSSG and $GS\dot{S}G^-$. $GS\dot{S}G^-$ formation is very probable, since in the cell the concentration of GS^- is very high as compared with $GS^{\cdot}$. Under the physiological conditions within erythrocyte metHb percentage is 0.3% – 1%; it corresponds to the concentration of $2\cdot10^{-5} - 3\cdot10^{-5}$ M. As it is shown in Fig. 5, those concentrations can lead to glutathione oxidation.

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Current Topics in Biophysics, vol. 18(2), 195-201

MODIFICATION OF RAT LIVER MEMBRANES BY ALDEHYDIC END-PRODUCTS OF LIPID PEROXIDATION

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(Accepted 28 December 1994)

The aldehyde end-products of lipid peroxidation, malondialdehyde (MDA) and 4-hydroxynonenal (HNE) modified rat liver membranes in a different manner. The preincubation of liver plasma membranes with MDA resulted in a significant decrease of the PGE_2 binding, whereas the membrane modification by HNE increased the amount of bound PGE_2. The rigidity of the internal region of membranes was enhaced under the influence of both MDA and HNE. Both MDA and HNE decreased the cytochrome P-450 content in rat liver microsomal membranes.

INTRODUCTION

Aldehydes derived endogenously during lipid peroxidation are an important damaging factor in oxidative stress. These aldehydic end-products generated as a result of the peroxidative breakdown of polyunsaturated fatty acids are very reactive compounds. They can damage cell structures localized distantly from the site of their origin. Among these aldehydes, malondialdehyde (MDA) and 4-hydroxyalkenals, especially 4-hydroxynonenal (HNE), are by far relevant products.

The cytotoxic effect of these aldehydes is mainly connected with the modification of different cell structures by their reaction with either SH-groups of low-molecular-weight compounds or proteins (Esterbauer, Schaur & Zollner, 1991; Dianzani, 1993) or by the interactions with ε-amino groups of proteins to form Schiff's bases. The toxic action of MDA and HNE can be related to supression of DNA and protein synthesis, inhibition of the mitochondrial respiratory chain, inactivation of several enzymes, etc.

Biomembranes structure conditions all biochemical events (translocation of molecules and ions, transmembrane signal transduction, membrane-bound enzyme activities) occurring at this level. Modification of membranes by various ligands induces different changes in membrane structure and functions, e.g. receptor redistribution and conformational changes, alterations in enzyme activities, changes in membrane physical parameters (fluidity, lipid structural order, hydrophobicity) and phospholipid turnover. Receptors of liver plasma membranes occupied by autonomic transmitters, prostaglandins (PG) or catecholamines, are responsible for transmembrane signalling, regulation of second messenger levels and metabolic changes in the hepatocyte resulting from activation of protein kinases. Cytochrome P-450 localized in microsomal membranes is responsible for detoxication of xenobiotics and metabolism of different endogenous substrates (eicosanoids, steroids, etc.). Alterations of functions of these proteins playing an important physiological role in living cells due to modification of membrane structures by aldehydic end-products of lipid peroxidation can lead, in turn, to significant metabolic disorders.

MATERIALS AND METHODS

Unlabeled PGE_2 were obtained from "Kemasol". (Tallinn, Estonia). ^{3}H-PGE_2 (140 Ci/mmol) was purchased from the Radiochemical Center (Amersham, England) and was chromatographed upon arrival against authenthic PG standard. All the spin probes were obtained from "Sigma" (USA). MDA was prepared from 1,1,3,3-tetramethoxypropane (" Aldrich", USA) by acid hydrolysis. HNE was a generous gift of Prof. H. Esterbauer (University of Graz, Austria).

Male rats weighing approximately 250 g were used for liver plasma and microsomal membrane preparation. The animals were decapitated after

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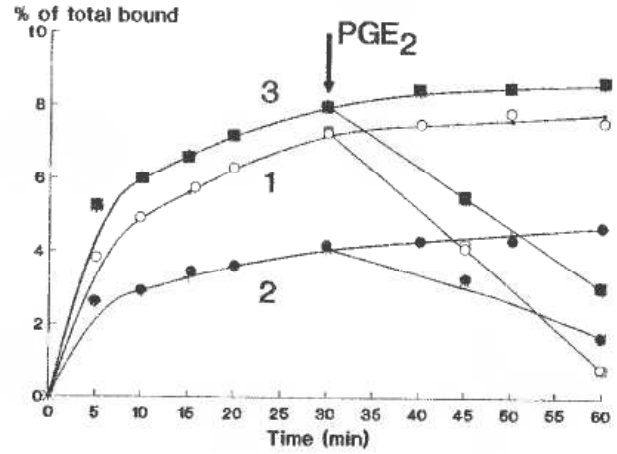

Fig. 1. Time course of binding of ^{3}H-PGE_2 to the liver plasma membranes and the dissociation of the ligand by an excess of unlabelled PGE and effect of the preincubation with MDA (2) and HNE (3). Membranes untreated by aldehydes were used as control (1). The 1000-fold excess of unlabelled PGE was added for the ^{3}H-PGE_2 dissociation.

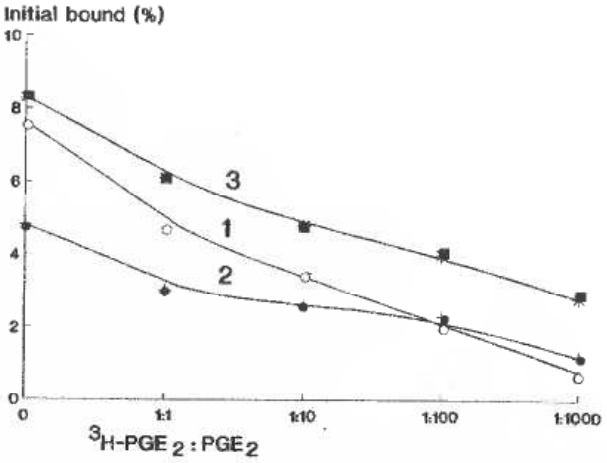

Fig. 2. Effect of excess of unlabelled PGE_2 (1:1 to 1000:1) on the binding of the ^{3}H-PGE_2 by native (1) and modified by MDA (2) or HNE (3) liver plasma membranes.

the liver perfusion in situ with 1 mM sodium bicarbonate. Plasma membranes and microsomes were isolated from whole liver homogenates as described elsewhere (Buko & Sushko, 1988; Buko & Zavodnik, 1990). Protein concentration was measured in membranes by the method of Lowry *et al.* (Lowry, Rosebrough, Farr & Randall, 1951). Cytochrome P-450 content was determined from the carbon monooxide difference spectrum upon dithionite reduction according to Omura and Sato (1964).

The PGE binding studies were carried out using a system containing 50 mM Tris-HCl buffer, pH 7.5, 1 mM $MgSO_4$ and 124 nM of ^{3}H-PGE_2. The total volume of the incubation medium was 350 µl. The binding was initiated by the addition of 50 µl of the plasma membrane suspension containing 165 µg of protein. The samples were incubated at 30°C in a shaking water-bath for 30 min. The binding was terminated by placing the tubes into an ice bath.

Membrane-bound ^{3}H-PGE_2 was precipitated by centrifugation in a microfuge for 15 min at 2200 x g at 5°C. Each estimation was an average of three experiments. The pellet was washed once with 1.0 ml of 50 mM Tris-HCl buffer, pH 7.5, containing 1 mM $MgSO_4$, resuspended in 1 ml of this buffer and transferred to a scintillation vial. The samples were counted in a Mark II liquid scintillation counter (Nuclear Chicago, USA), using an universal cocktail. Radioactivity of free ^{3}H-PGE_2 was counted in a supernatant in a similar manner.

The binding data were transformed and plotted according to Scatchard (1949). The competition for ^{3}H-PGE_2 binding sites was performed under the same conditions with unlabelled PGE_2 added in an 1- to 1000-fold excess. The time course and reversibility of the ^{3}H-PGE_2 binding were studied using membranes incubation during 60 min. To some reactions tubes 1000-fold excess of unlabelled PGE_2 was added after 30 min. of incubation.

Liver plasma membranes were modified by MDA and HNE in 50mM Tris-HCl buffer, pH 8.0 at 22°C for 4 h. Preincubation of liver microsomal membranes with MDA or HNE as well as with aliphatic aldehydes or p-chloromercuribenzoate (PCMB) was performed in the above buffer at 22°C during 5 min. Cytochrome P-450 content

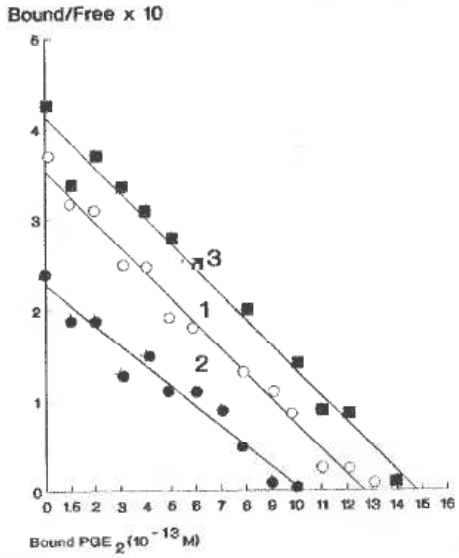

Fig. 3. Scatchard analysis of ^{3}H-PGE$_2$ binding to native (1) and MDA (2) or HNE (3) modified liver plasma membranes.

was measured after preincubation of microsomes with MDA or HNE during 5 min.

All the ESR spectra were taken at room temperature (22°C) on ERS-220 spectrometer (Germany). The usual instrumental parameters were: microwave power 5 mW; center of the field 3300 G, scan range 100 G. Quartz tubes of 1 mm internal diameter were used. 5-, 12 and 16-doxyl derivatives of stearic acid were used for a transversal scanning of liver plasma membranes. Spin label modifying SH-groups of membrane protein, 1-oxyl-2,2,6,6,-tetra-methylpiperidyl-PCMB (OTMP-PCMB) was also used in the experiment. The rotational correlation time and the lipid bilayer structural order were calculated using a routine method (Griffith & Jost, 1979).

RESULTS AND DISCUSSION

Effect of MDA and HNE on prostaglandin E_2 (PGE_2) receptors of liver plasma membranes

We studied the effect of MDA and HNE on the binding of PGE_2 to liver plasma membranes. The specific binding of PGE_2 to membranes increased in a time-dependent manner (Fig. 1). The preincubation of liver plasma membranes with MDA resulted in a significant decrease of the ^{3}H-PGE_2 binding, whereas the membrane modification by HNE increased the amount of bound tritiated PGE_2. The total binding of ^{3}H-PGE_2 to liver plasma membranes was significantly less pronounced in MDA-modified membranes than in native ones, whereas the nonspecific binding was approximately the same in both the modified and native membranes (Fig. 2). The alkylation of liver plasma membranes by HNE slightly enhanced the total binding of of ^{3}H-PGE_2 but dramatically increased the nonspecific binding to membranes.

Recently we have shown that rat liver plasma membranes contained a single class of receptors for PGE_2 (Buko, 1991). The modification of membranes by MDA led to a decrease of the K_{ass} for ^{3}H-PGE_2 and slightly reduced the PGE_2 receptor density, whereas the preincubation with HNE enhanced the receptor density but did not essentially change the K_{ass} (Fig. 3).

The effect of MDA and HNE on the structural profile of liver plasma membranes was investigated by ESR spectroscopy using doxyl derivatives of stearic acid (DSA) as spin probes. MDA increased the rotational correlation time of 5-DSA whose nitroxyl radical was located in the surface zone of membrane, whereas the alkylation of membranes with HNE decreased this parameter (Fig. 4). Both MDA and HNE increased the rotational correlation time of 12-DSA with the doxyl group immersed in deeper areas of the lipid bilayer (Fig. 5).

The spin label behavior indicated that the modification of liver plasma membranes by the aldehydes changed the membrane microviscosity. The rigidity of the internal zone of membrane leaflets as assesed by the 12-DSA behavior was enhanced under the influence of both MDA and HNE. However, the microviscosity of the surface zone was increased following MDA modification and was diminished after the HNE alkylation.

The observed changes in the functional parameters of the ^{3}H-PGE_2 binding by liver plasma membranes following the modification by MDA or HNE were related to structural alterations in the membrane characterized by changes in the profile of its rigidity. This, in turn, depends on the aldehyde properties, particularly on their hydrophobicity, and is governed by the aldehyde localization in the membrane.

ESR study on MDA and HNE interactions with liver microsomal membrane

The changes in the liver microsomal membrane structure are closely related to the alterations of cytochrome P-450 functions. The decrease of the cytochrome P-450 content is usually associated with its transformation to the inactive form, cytochrome P-420 (Schenkman, Remmer & Estabrook, 1967).

The modification of rat liver microsomal membranes led to a decrease of the cytochrome P-450 content. Figure 6 shows a dependence of the cytochrome P-450 content on the concentration of MDA and p-chloro-

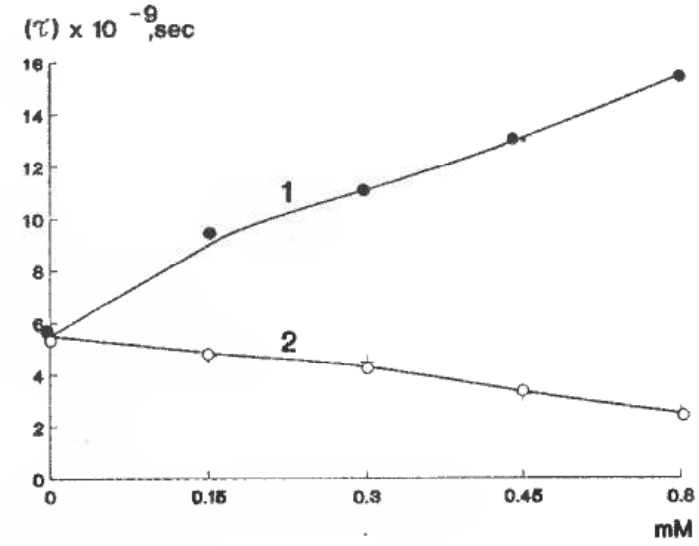

Fig. 4. The effect of MDA (1) and HNE (2) on the rotational correlation time (τ) of a lipid spin probe, 5-DSA, bound to liver plasma membranes

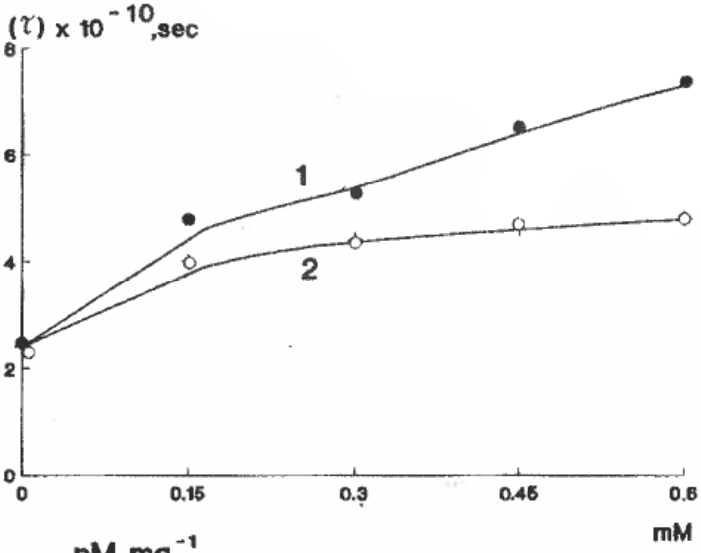

Fig.5 The effect of MDA (1) and HNE (2) on the rotational correlation time (τ) of a lipid spin probe, 12-DSA, bound to liver plasma membranes.

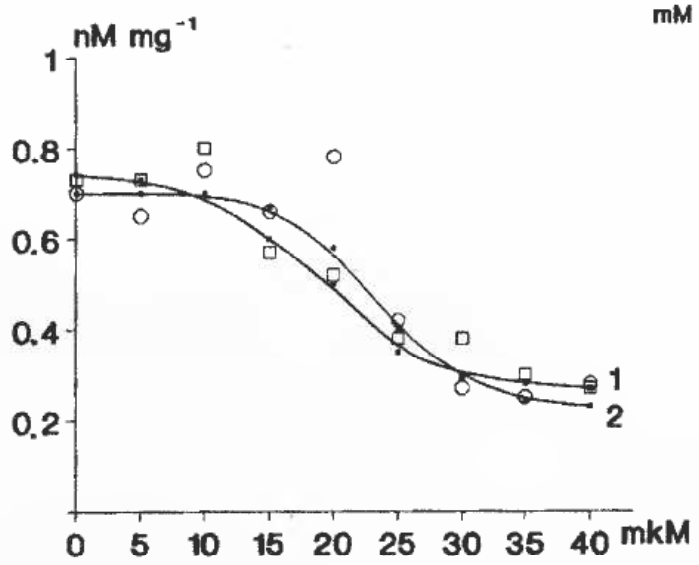

Fig.6. Decrease of the content of cytochrome P-450, measured by the CO-difference spectra, in rat liver microsomes after preincubation with PCMB (1) and MDA (2).

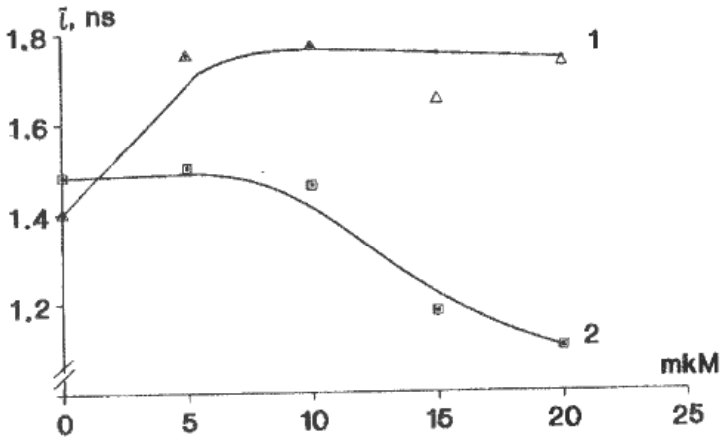

Fig.7. Effect of HNE (1) and MDA (2) on the rotational correlation time (τ) of the spin label OTMP-PCMB bound to SH-group of liver microsomes.

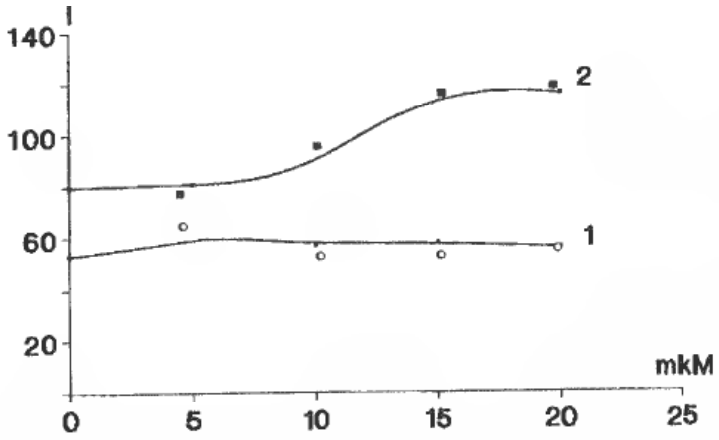

Fig.8. Effect of HNE (1) and MDA (2) on the intensity of ESR signal (I) of spin label OTMP-PCMB.

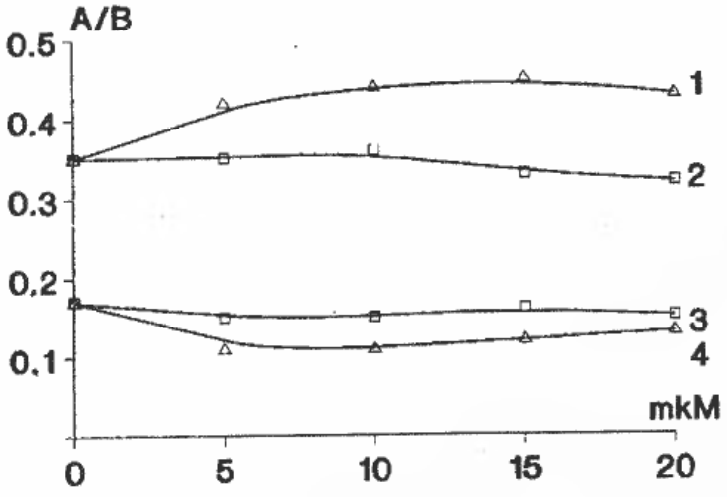

Fig.9. Effect of HNE and MDA on the lipid structural order evaluated by lipid spin probes, 5-DSA (1 – HNE; 2 – MDA) and 16-DSA (3 – MDA; 4 – HNE) bound to rat liver microsomal membranes.

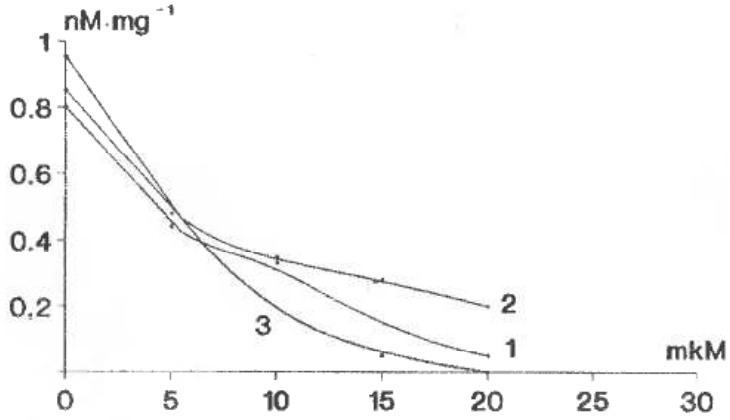

Fig. 10. Decrease of content of cytochrome P-450, measured by the CO-difference spectra in rat liver microsomes after preincubation with HNE (1), caprylic aldehyde (2) or valeric aldehyde (3).

mercuribenzoate (PCMB), a specific reagent modifying SH-groups. MDA did not affect the cytochrome P-450 content in rat liver microsomes at concentrations lower than 10 μM whereas an increase in the MDA concentration from 10 μM to 20 μM dramatically decreased the cytochrome P-450 content down to a plateau at the MDA concentration higher than 20 μM. The curve of microsome titration with PCMB is practically similar to the graph obtained by titration with MDA.

PCMB inactivate cytochrome P-450 due to its conversion to cytochrome P-420, modifying SH-groups of the hemoprotein (Lyakhovich & Tsyrlov, 1978). The similarity of the disposition of the both curves obtained with MDA and PCMB titration can indicate a common mechanism of cytochrome P-450 inactivation for the both reagents. It is possible that modification of cytochrome P-450 SH-groups by MDA led to a decreased cytochrome P-450 content via its conversion to cytochrome P-420, similar to PCMB modifiation.

This assumption is confirmed by a decrease of the rotational correlation time (τ) of the spin label OTMP-PCMB bound to SH group of the microsomal membrane at the same concentrations of MDA (10-20 μM). Figure 7 shows that these concentrations of MDA dramatically lowered microviscosity of the lipid surrounding of this label.

The spin label, OTMP-PCMB specifically binds to SH-groups and MDA at the above concentrations (10-20 μM) very intensively competes with the spin label for SH-groups of microsomal proteins increasing the concentration of free spin label (Fig. 8).

MDA slightly decreased the order of membrane lipid bilayer in the presence of spin probes, 5-doxylstearic acid (DSA), with the nitroxyl radical being localized in the surface zone of membrane and 16-DSA with the nitroxyl radical situated in the deeper zone of the lipid bilayer (Fig. 9).

HNE dramatically decreased the cytochrome P-450 content of rat liver microsomes at concentrations lower than 10 μM whereas this hemoprotein was not detected at HNE concentrations of 10 μM and higher (Fig. 10). Aliphatic aldehydes, valeric and caprilic, decreased the cytochrome P-450 content in a similar manner.

The microviscocity of the lipid environment of the spin label OTMP-PCMB, bound to SH-groups of membrane protein is decreased under the influence of HNE (Fig. 7). At the same time we did not observe a release of the free label from the binding sites due to competition with HNE as seen for MDA (Fig. 8).

The order of membrane lipid bilayer was significantly increased by HNE in the presence of 5-DSA (Fig. 9), which indicates relaxation of protein-lipid interactions in the area corresponding to the location of the 5-DSA nitroxyl radical. Simultaneously HNE did not practically change the behavior of 12-DSA and 16-DSA whose doxyl group are located in deeper zones of microsomal membranes.

The heme of cytochrome P-450 is immersed into microsomal membrane of a depth of $4 \cdot 10^{-10}$ m (Kulikov, Cherepanova, Likhtenshtein, Uvarov & Archakov, 1989) at which the doxyl group of 5-DSA in located. Therefore, we can assume that the inactivation of cytochrome P-450 by HNE is related to disturbances of hydrophobic protein-lipid interactions in this area of the microsomal membrane. Some authors believe that relaxation of protein-lipid interactions is the main mechanism of cytochrome P-450 conversion to cytochrome P-420 (Lyakhovich, Tsyrlov, 1978).

The above data indicate different mechanisms of the MDA and HNE interactions with microsomal membranes and different effects of these aldehydes on the cytochrome P-450 content. The effect of MDA on the latter parameter is related to modification of protein microsomal SH-groups,

whereas the effect of HNE is mediated by disturbances of the hydrophobic interactions between the cytochrome P-450 and the phospholipid surrounding of this hemoprotein in the surface zone of the microsomal membrane where the heme of this hemoprotein is located.

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Current Topics in Biophysics, vol. 18(2), 202-204

FREE-RADICAL METHOD FOR L-ASCORBIC ACID DETERMINATION IN TISSUES OF EXPERIMENTAL ANIMALS

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(Accepted 28 December, 1994)

Tissues of living organisms, lyophilized and adequately prepared, in air conditions reveal a strong signal of electron paramagnetic resonance. In vitro it originates from ascorbyl free radical, whereas its precursor in vivo is only L-ascorbic acid. This served to develop a new free-radical method for determining vitamin C and, indirectly, free radical oxidative processes contents in animal tissues. Under strictly defined circumstances the ESR signals are reproducible, and this method is sensitive and valuable.

INTRODUCTION

L-ascorbic acid (vitamin C) is an important substance in the biochemistry of living organisms. It acts either as reductant or cofactor in certain enzymatic reactions and is a component of general antioxidant buffer system. For a long time it has been known to play an apparent role in scurvy (Hodges, 1976), in collagen synthesis (Udenfriend, 1966), peroxidations of lipids (Haase & Dunkeley, 1969), control of mitotic activity (Edgar, 1970), antimutagenic activity (Norkus & Kuenzig, 1985), biologic transport (Lohmann, 1984), as well as in neoplasms (Lohmann, Schreiber, Strobelt & Muller-Eckhardt, 1979). The level of L-ascorbic acid in solutions or body fluids is defined most frequently by making use of its reducing properties, with various oxidizing factors being implemented (Verma, 1982; Jarzembiński & Ługowska, 1989; Bączyk & Świdzińska, 1970). The determination of its content in tissues encounters a lot of obstacles due to the presence of substances, whose redox potentials are similar to the potential of the system: ascorbic acid/dehydroascorbic acid. This study furnishes a proposal for the introduction of a new method of estimating the L-ascorbic acid content in tissues of laboratory animals, which is called a "free radical method".

MATERIAL AND METHOD

The tissues (ca 200 mg) are taken from the animals studied under ether narcosis. They are subjected to lyophilization (lyophilizator LGA 05) or dried in vacuum (Unitra rotary pump) at a temperature not higher than 310 K. Then the tissues are ground in small porcelain evaporators, and, after a lapse of 1 hour, placed in 20 mg portions, in quartz tubes 5 mm in diameter. The tube with the studied tissue is put into the resonance chamber of ESR spectrometer, and its microwave spectrum is recorded. ESR spetrometer, type SE/X 2544 (Radiopan) with resonance chamber RCX 660 was used in the studies. Measuring conditions were: frequency 9.45 GHz, power 3 mW, modulation 100 kHz/0.2 mT, time constant 1s, scan rate 50 mT/4 min. DPPH was employed as a standard to determine the g coefficient and the concentration of unpaired spins. ESR spectra were measured in various tissues (blood, kidneys, lungs, spleen, liver) of mice, guinea-pigs and rats, as well as in adrenals of rats. The animals stemmed from own breeding stock and in the course of the studies they were middle-aged, of different weight and of both sexes. Care must be taken to secure strict reproducibility of conditions at samples preparations (oxygen tension, humidity, temperature) and ESR spectra measurements. ESR signal maximum occurs after the lapse of 1 h upon termination of lyophilization process (when the measurements were made); after 24 hours the amplitude of the signal decreases by about 30 %.

RESULTS

As an example (Fig. 1) ESR signals obtained from mouse tissues are given. All the signals display the same shape and location (ΔB_{pp} = 0.8 mT, g = 2.005), and they differ only with respect to amplitude, which denotes different concentration of un-

Table 1. Concentration of unpaired spins (units: $\times 10^{16}$/gram) in dried tissues of mice, guinea-pig, and rats ($x \pm SD$)

Tissues	Mice	Guinea-pig	Rats
Blood	1.22 ± 0.25	0.70 ± 0.24	1.46 ± 0.35
Kidneys	2.30 ± 0.57	1.94 ± 0.46	3.23 ± 0.90
Lung	4.62 ± 0.62	3.79 ± 0.55	4.75 ± 1.59
Spleen	5.28 ± 0.82	3.84 ± 0.98	6.74 ± 1.57
Liver	4.18 ± 0.75	1.80 ± 0.36	4.46 ± 0.64
Adrenals			28.1 ± 6.9

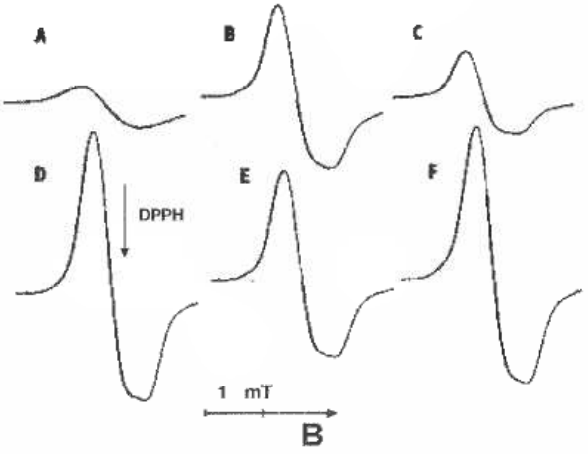

Fig. 1. The ESR spectra of mouse tissues: blood (A), heart (B), kidneys (C), liver (D), lung (E), spleen (F).

coupled spins in the samples studied. That finding refers also to the tissues of guinea-pigs and rats. The number of unpaired spins in tissues of all the studied animals calculated in units $\times 10^{16}$/gram (dry tissue) is given in Tab.1 ($x \pm S$ – mean ± standard deviation for 10 animals).

Particularly strong signals were shown by adrenals of the rats. Tenfold determinations of ESR signals from the liver of one rat permitted estimation of the error of the method, equal to ± 4 %.

DISCUSSION

As shown by previous studies (Dodd & Swartz, 1980; Lohmann & Neubacher, 1984) the type of free radical detected by ESR technique depends on the way the samples have been prepared. Lyophilization or drying of tissues in vacuum at the temperature not higher than 310 K, followed by exposure of samples to the air, and the recording of ESR spectra at room temperature disclose a strong signal with parameters: $\Delta B_{pp} = 0.8$ mT, $g = 0.005$; the source of the signal is the ascorbyl free radical (Mueller & Tannert, 1986; Natkinis & Cerniauskiene, 1974). Under *in vivo* conditions it corresponds to the concentration (level) of L-ascorbic acid in the tissues (Cimbolairtyte *et al.* 1982). ESR signals seen in the presented experiments originated from that radical. Hence, the level of L-ascorbic acid in the tissues is proportional to the amount of free radicals (uncompensated spins) determined by this method. Only the signal from the blood was somewhat wider ($\Delta B_{pp} = 1$ mT), because its source apart from ascorbic acid, may be vitamin P (Cimbolaityte *et al.*, 1982). The study on the dependence of the amount of unpaired spins X as a function of ascorbate added to the blood reveals a linear dependence up to ascorbate concentration of 100 mM. The presented method of determining L-ascorbic acid content in tissues of laboratory animals may most adequately be employed for its relative determination; X should be considered as arbitrary unit. An attempt can also be made to define the absolute concentration of ascorbate. Basing on the assumption that 1 mol of each substance contains $6 \cdot 10^{23}$ molecules, while one molecule of ascorbic acid may potentially possess 1 unpaired spin, up to 10^{16} of spins stem from $3 \cdot 10^{-3}$ mg of vitamin C (MW ~ 176). In recalculation for 100 g of dry tissue it corresponds to a concentration of 0.3 mg/100g. The vitamin C concentration calculated from Tab. 1 in absolute units is smaller as compared with the results obtained by other methods, Horning (1975).

However, it should be found out whether there is 100 per cent "yield", it means, if each vitamin C

molecule in the tissue would render, in consequence of sample preparation procedure, its own contribution to ESR signal. For this purpose, a known vitamin C amount should be added to the blood; measurements and calculations should be performed according to the mentioned method, which will facilitate the calculation of normalized coefficient of the yield (from 1 to 0) and correction (multiply by reciprocal of the coefficient) of vitamin C concentration in absolute units. The establishing of the coefficient as well as other aspects of such determination will be the subject of a separate paper. On many occasions it would be sufficient to define the relative values of vitamin C level in the tissues. This method was referred to in a number of own papers (Gonet, 1989; Wójcicki, *et al.* 1989, 1994).

It is known (Buettner, 1993), that the ESR method may be implemented in direct detection of ascorbyl free radical in vivo, which arises from L-ascorbic acid under the influence of free radical oxidative processes ($R^{\cdot} + AH \rightarrow A^{\cdot} + RH$). As free radical processes increase in a system, the steady-state $A^{\cdot}$ concentration rises too. The ESR intensity of this radical $A^{\cdot}$ signal can serve as an indicator of the degree of free radical processes. In my investigations the process is studied by measuring substrate AH (rel. unit), but not the product $A^{\cdot}$. The drop of EPR signal *in vitro* samples denotes the diminution of substrate content *in vivo*, which may result from the intensity of free radical reactions or other mechanisms, causing substrate depletion.

CONCLUSION

A new, free radical, method of determining L-ascorbic acid content in tissues of laboratory animals has been presented. The accuracy of the method for relative determinations is ± 4 %. The method is suitable to be used in different laboratory studies e.g. pharmacological, ecological and dietary, and in studies on the influence of ionizing radiation on living organisms (vitamin C being a scavenger of free radicals). The widespread use of ESR spectroscopy makes the proposed method accessible to a considerable extent.

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Current Topics in Biophysics, vol. 18(2), 205-207

SENSITIVITY OF TRISOMIC-21 HUMAN FIBROBLASTS TO GAMMA-RADIATION AND CARMINOMYCIN

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(Accepted 28 December 1994)

Relation between activity of antioxidant enzymes superoxide dismutase (SOD),catalase, glutathione peroxidase (GSH-Px) and sensitivity of human cells displaying different intracellular level of these enzymes, to the damaging effect of ionizing radiation and carminomycin has been investigated. Six different cell lines of normal and trisomic-21 fibroblasts were used. All trisomic fibroblast cell lines display the 50% increase of SOD activity and 30% of GSH-Px activity. It has been shown that effect of carminomycin on trisomic-21 fibroblasts is weaker compared to that on normal fibroblasts. At the same time trisomic-21 fibroblasts are more sensitive to gamma radiation despite of higher intracellular activity of SOD and GSH-Px. These results indicate that different mechanisms are responsible for sensitivity of normal and trisomic-21 cells to ionizing radiation and carminomycin.

INTRODUCTION

Anthracycline antibiotics belong to the group of drugs known to be effective in a wide variety of human malignancies. Although precise mechanism of cytocidal action of these drugs is still unknown, three possible mechanisms are considered at present: binding to DNA and inhibition of biochemical processes regulated by DNA, interaction with cell membranes leading to disturbance in transport through the membrane as well as production of the superoxide radical anion and other reactive oxygen species (Powis, 1989; Peskin, Koen, Zbarsky & Konstantinov, 1977).

Semiquinone forms of anthracyclines are activated by microsomal, nuclear envelope and plasma membrane electron transport chains in cells, where they react with a molecular oxygen yielding superoxide radical anions $O_2^{\cdot}$ (Peskin & Bartosz, 1987; Bachur, Gee & Friedman, 1988; Land, Mukherjee, Swallow & Bruce, 1983). Peroxide adducts of carbon-centered DNA radicals which decomposition may lead to the single and double strand breaks of DNA have also been observed (Krugh, 1994). As a result of all these processes reduction of viability occurs, which in turn may result in cell death.

Enzymatic systems involving superoxide dismutase (SOD), catalase, glutathione peroxidase (GSH-Px) and low-molecular weight antioxidants are currently considered to be the main protective mechanism against damaging effects of active oxygen species generated in cells. Assuming that cytotoxicity of anthracycline antibiotics is mediated by reactive oxygen species, similarly to ionizing radiation, it can be expected that cells having naturally elevated levels of some antioxidants should respond in a different way to these antibiotics compared to normal cells. Human cells with trisomy-21, which display a 50% higher activity of Cu,Zn-superoxide dismutase by gene dosage effect in this chromosome, seem to be a good model for studies on the mechanism of anthracycline antibiotics action as well as the role of antioxidant enzymes in this process (Rózga, Peskin & Bartosz, 1990).

The aim of this work was to investigate relation between activity of enzymes involved in the decay of water radiolysis products and sensitivity of human cells, displaying different levels of antioxidant enzymes, to the damaging effect of ionizing radiation and carminomycin. Damage to the cells was estimated on the basis of their viability measured by MTT test and cell survival tested by the colony forming ability.

Normal donor skin fibroblasts and trisomic-21 donor foetal tissue fibroblasts were used in this study. Cells were obtained from The Center of Child Health, Warsaw, Poland (normal: S-126, trisomic: T-74, T-107, T-155, T-158) and from Institute of Medical Genetics of the Academy of Medical Sciences, Moscow, Russia (normal: B-2, trisomic: B-2). All cell lines were routinely maintained as a monolayer culture in Eagle MEM me-

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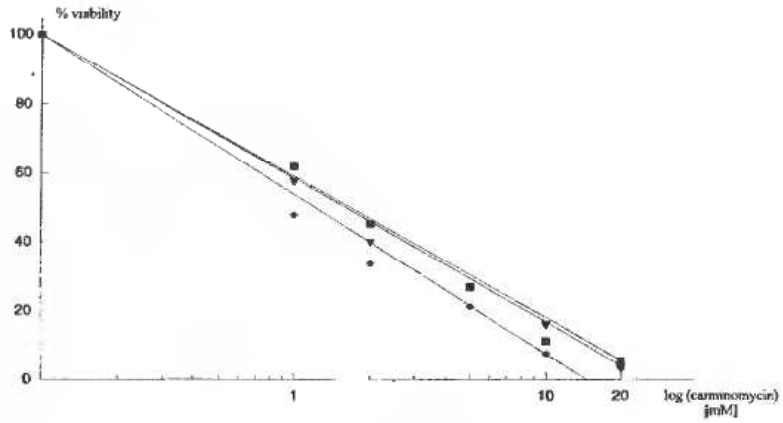

Fig 1. Viability of human fibroblasts treated with carminomycin. Cell lines: diamonds – S-126; squares – T-155; triangles – T-158.

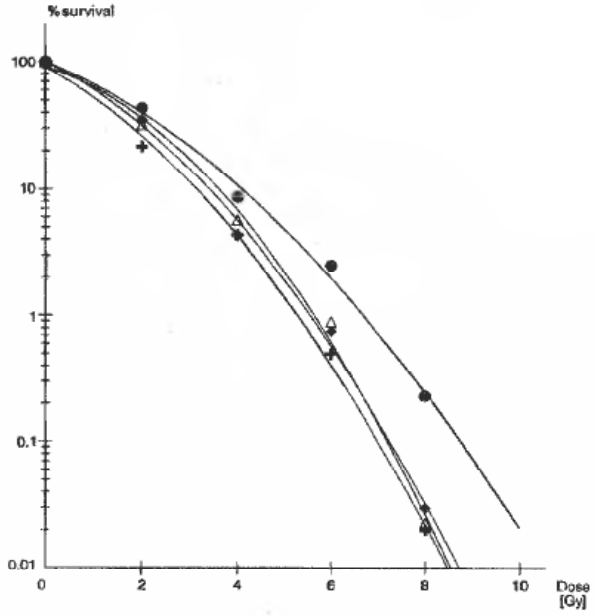

Fig 2. Survival of gamma-irradiated fibroblasts. Cell lines: circles – B-2; diamonds – B-1; empty triangles – T-74; crosses – T-107.

dium supplemented with a 10% heat-inactivated newborn calf serum and 15% lactalbumin hydrolysate in humidified 5% CO_2 at 37°C. Gentamycin was added at a concentration of 5 µg/cm^3 medium. Cells between 5 th and 15 th passage were used in all experiments.

Activity of SOD was estimated in cell homogenates by inhibition of NBT reduction in a xanthine-xanthine oxidase system (Beauchamp & Fridovich, 1971) after removing cell membranes by centrifugation of homogenates at 110 000×g. Activities of catalase and GSH-Px were estimated according to the method of Aebi (1974) and Hopkins and Tudhope (1973), respectively. Cell viability was estimated by MTT test (Mosmann, 1983), cell survival by colony forming ability assay (Paterson, Anderson, Smith & Smith, 1979).

All trisomic-21 fibroblast cell lines display the 50 % increase of SOD activity and about 30% of GSH-Px activity.

On the basis of cell viability test and cell survival curves it has been shown that effect of carminomycin on trisomic-21 fibroblasts is weaker compared to that on normal fibroblasts. On the other hand trisomic-21 fibroblasts are more sensitive to gamma-radiation which indicates the

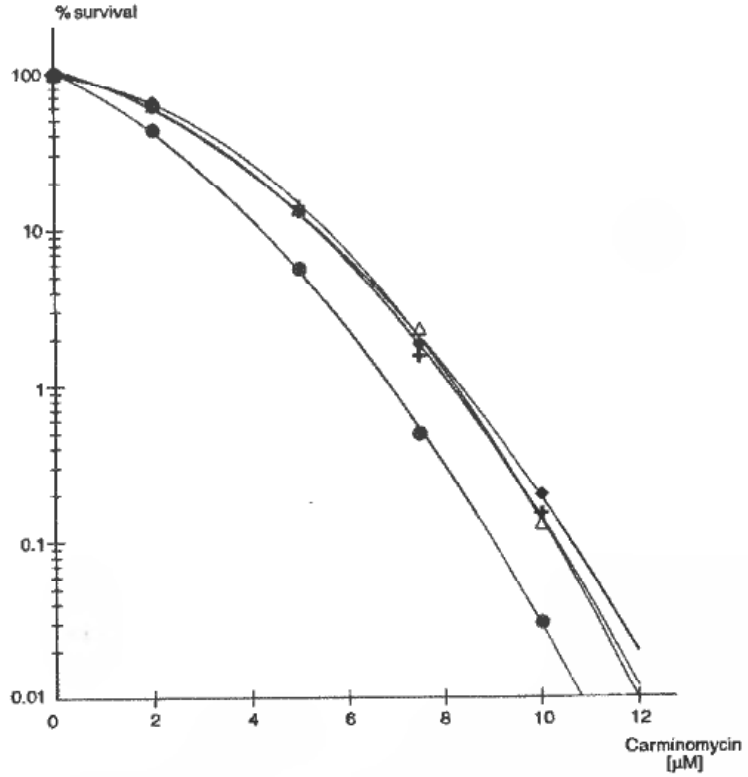

Fig 3.Survival of human fibroblasts treated with carminomycin. Cell lines: circles – B-2; diamonds – B-1; empty triangles – T-74; crosses – T-107.

greater number of unrepaired DNA double-strand breaks leading to the reproductive death of cells. If seems that higher level of antioxidant enzymes in trisomic-21 cells may contribute in significant degree to cell resistance to the action of carminomycin.

On the contrary, these enzymes, mainly SOD, do not reveal any protective effect on trisomic-21 cells subjected to gamma-irradiation.

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Current Topics in Biophysics, vol. 18(2), 208-210

THE COMPARISON OF PRO- AND ANTIOXIDANT PROCESSES AND ACTIVITIES OF LIPID METABOLISM IN THE RABBITS ON CASEIN AND SOYBEAN PROTEIN ENRICHED DIETS

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(Accepted 28 December 1994)

The role of some animal proteins in the induction of atherosclerosis is known from many years, but the mechanism of atherogenesis through dietary proteins is not understood. It has been known from many years that dietary proteins influence the cholesterol metabolism, but the metabolic basis for the effects of dietary proteins on cholesterolemia is controversial. Casein is the best known atherogenic protein. It is rich in methionine, which is metabolised to homocysteine. Homocysteine and its derivatives have atherogenic effect. Earlier we have found that methionine in rabbit diet changes lipid peroxidation and antioxidant activity, so it was interesting to test the effects of casein-enriched diet on free radicals/ lipid peroxidative and antioxidant processes, as well as on lipid metabolism. Prostherogenic profil of serum lipids, increased level of lipids in the aortas and decreased activities of all researched enzymes involved in lipid metabolism [total postheparin plasma lipolytic activity, hepatic triglyceride lipase, lipoprotein lipase, acyltransferase lecithin: cholesterol], were observed in the animals on the casein enriched diet in comparison with the animals fed the soybean protein diet. Increased activities of antioxidant enzymes in aortas [catalase, superoxide dismutase, glutathione peroxidase], decreased serum antioxidant activity and concentration of ceruloplasmin in the rabbits on the casein enriched diet are pointing to the participation of free radical/ antioxidant processes in the induction of atherosclerosis in this model.

INTRODUCTION

The role of animal proteins in induction of atherosclerosis is known from the beginning of XX-th century, but the mechanism of atherogenesis through dietary proteins is not fully understood. It has been known from many years that dietary proteins influence cholesterol metabolism, but the metabolic basis for the effects of dietary proteins on cholesterolemia is controversial (Kopieczna-Grzebieniak, Piskorska, Łowińska-Ogonowska, 1992). The most often studied dietary proteins were casein and soybean protein (Sugano & Koba, 1993; Noguchi, Takita, Suzuki, Nakamura & Inami, 1992). Casein is a protein rich in methionine. The metabolites of this amino acid, as homocysteine and its derivatives, have atherogenic effects (Olszewski & McCully, 1993; Wilcken & Dudman, 1992). Earlier we have found that methionine in rabbit diet changes lipid peroxidation and antioxidant activity (Toborek, 1989), so it was interesting to test the effects of casein enriched diet on free radicals/lipid peroxidative and antioxidant processes as well as on lipid metabolism.

MATERIALS AND METHODS

Twenty male, New Zealand rabbits (3 months old) were divided into 2 groups: casein group, which diet was enriched with casein (Murowana Goślina, Poland) and control group, which diet was enriched with the same amount of soybean protein prepared by the extraction of soybean (Rolimpex, Kochanowice, not far from Lubliniec, Poland). The animals were fed the experimental diets for 7 months. The protein preparations, which were used to this experiment, were not free from fat. The measured amount of total lipids was 2% in the casein preparation used and 4.5% in the soybean protein preparation. To eliminate the influence of these lipids on the measured parameters soybean oil in the amount of 1.8 % was added into the diet of casein group and butter in the amount of 0.8 % was added into the fodder of soybean protein (control) group. The proportion of standard fodder for the rabbits (Fodder Manufactures, Motycz, Poland) and the preparation of proteins (casein or soybean) was 3:2.

After 7 months of feeding experimental diets lipid peroxidation was estimated as TBARS

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Table 1. Changes of body mass, of lipids metabolism parameters in serum and aorta homogenates, and some activities of lipid metabolism enzymes (postheparin plasma lipolytic activity – PHLA, hepatic triglyceride lipase – HL, lipoprotein lipase – LPL, acyltransferase lecithin: cholesterol – LCAT) in serum rabbits of fed casein-enriched diet for 7 months.

Measured parameter	Groups	
	Casein group	Soy protein group
Body mass (kg)	3.25 ± 0.20	3.30 ± 0.23
Total cholesterol (mmol/l)	1.76 ± 0.41*	0.44 ± 0.13
Free cholesterol (mmol/l)	0.56 ± 0.26**	0.18 ± 0.03
Cholesterol esters (mmol/l)	1.20 ± 0.15*	0.26 ± 0.10
Cholesterol LDL (mmol/l)	1.11 ± 0.23*	0.22 ± 0.02
Cholesterol HDL (mmol/l)	0.63 ± 0.19*	0.20 ± 0.01
Phospholipids (mmol/l)	2.65 ± 0.41**	1.88 ± 0.14
Triglycerides (mmol/l	0.50 ± 0.07	0.42 ± 0.14
PHLA (μmol/h/ml)	35.15 ± 6.06**	55.32 ± 6.97
HL (μmol/h/ml)	10.04 ± 1.59**	17.25 ± 2.61
LPL (μmol/h/ml)	25.11 ± 5.63*	38.06 ± 4.55
LCAT (μmol/h/l)	29.90 ± 13.50**	51.20 ± 13.10
Cholesterol in aorta (μmol/mg of protein)	1.43 ± 0.27	1.50 ± 0.31
Triglycerides in aorta (μmol/mg of protein)	35.17 ± 4.29*	27.03 ± 3.89

Table 2. Changes of aorta thiobarbituric acid reactive substances concentrations (TBARS) and antioxidant enzymes activities in aorta: catalase (Cat), superoxide dismutase (SOD), glutathione peroxidase (GPx), antioxidant activity of serum (AOA) and serum ceruloplasmin concentration in the rabbits fed casein-enriched diet for 7 months.

Measured parameter	Groups	
	Casein group	Soy protein group
Cat (k/sec/g of protein)	79.0 ± 14.0*	12.0 ± 7.1
SOD (U/mg of protein)	63.8 ± 7.5**	35.7 ± 4.6
GPx (U/g of protein)	29.4 ± 1.2**	24.7 ± 3.6
TBARS (μmol MDA/l of serum)	2.9 ± 0.5	2.3 ± 0.4
AOA (%)	21.6 ± 4.3**	29.7 ± 3.4
Ceruloplasmin (mg/l of serum)	165.4 ± 45.2*	321.0 ± 83.0

(thiobarbituric acid reactive substances) according to Michara, Uchiyama and Fukuzawa (1980). Superoxide dismutase activity (SOD) was determined according to Misra and Fridovich (1972) and catalase (CAT) according to Aebi (1983). Glutathione peroxidase (GPx) activity was measured by the method of Lawrence and Burk (1976), serum antioxidant activity (AOA) according to Stocks, Gutteridge, Sharp & Dormandy, 1974) and serum ceruloplasmin concentration by the method of Richterich (1971). Protein was measured by the modification of the Lowry method, Hartree (1972).

One unit of AOA is defined as a percentage of the inhibition of spontaneous autoxidation of rat brain homogenate. A unit of SOD is the quantity of the enzyme, which is required to produce 50% inhibition of the rate of conversion of epinephrine to adrenochrome. One unit of catalase is defined as the rate constant (k) of a first order reaction catalyzed by the enzyme. One unit of GPx is the amount of μmol oxidized NADPH/min.

Serum total cholesterol and cholesterol in the fractions of lipoproteins were estimated with Merck test kits, free cholesterol with a bioMericaux kit, triglycerides and phospholipids were determined enzymatically with test kits (Boehringer – Mannheim). Lipids in the aorta wall were estimated according to the following procedure: 20% homogenates were centrifuged (2000g, 10 min.). The pellets were extracted with the mixture of methanol-chloroform (1:2 v/v). The supernatants were evaporated and the dry residues were dissolved in ethanol. The concentrations of cholesterol and triglycerides in the extracts were estimated as in the serum.

Total postheparin plasma lipolytic activity (PHLA) was measured by the method of Kaneki *et*

al. (Kaneki, Nakauchi & Tanaka, 1987), released fatty acids were estimated according to Duncombe (1964), hepatic triglyceride lipase (HL) was measured by the method of Nozaki *et al.* (Nozaki, Kubo, Matsuzawa & Taruis, 1984). The activity of the lipoprotein lipase (LPL) was the residue between total lipolytic activity and hepatic lipase. Acyltransferase lecithin: cholesterol activity (LCAT) was measured with LCAT-test Praha (Institute for Research, Production and Application of Radioisotopes).

The data were analysed using the nonparametric Mann-Whitney U test. The values represent the means ± SEM of 10 animals (* and ** mean that the values between control and casein groups are significantly different: ** $p<0,01$; * $p<0,05$).

RESULTS

The mean body weights of the rabbits in CAS group were not statistically changed in comparison to those of the control group (Table 1). The levels of serum total, free and esterified cholesterol, LDL and HDL cholesterol and phospholipids concentrations in CAS group increased markedly. Triglyceride level in serum did not change clearly in CAS group in comparison with control (soy protein) group. The activities of all studied enzymes connected with lipids metabolism were decreased.

AOA and ceruloplasmin concentration in serum of the animals on casein diet was decreased (Table 2). The activities of SOD, CAT and GPx in aorta homogenates were increased in casein group, whereas increased TBARS concentration was not statistically significant.

CONCLUSIONS

Casein in the diet is an atherogenic protein to the rabbits: proatherogenic serum lipid profile, lipid changes in the aortas and decreased activities of enzymes connected with lipoprotein metabolism were observed in the animals on the casein enriched diet.

In the model of atherosclerosis induced with casein eriched diet, free radicals processes, at least in part, may be resposible for atherogenic effects:

a) decreased antioxidant activity of serum and ceruloplasmin concentration in serum as well as

b) increased activities of main antioxidant enzymes in aorta homogenates of these animals in comparison with the rabbits on soy protein group.

The increase of lipid peroxidation in serum of the rabbits on casein rich diet was not statistically significant.

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Current Topics in Biophysics, vol. 18(2), 211-215

OZONE-INDUCES CHANGES IN ^{1}H NMR SPECTRUM OF LIPOPROTEINS OF RABBITS WITH ATHEROSCLEROSIS

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(Accepted 28 December 1994)

The effect of free radicals and active forms of oxygen is connected with the pathogenesis of some diseases. Ozone is one of the forms of oxygen. When it is inhalated, it is toxic to the lung tissue. The mechanism of ozone acting on an organism, when it is administrated into the blood vesseis or into the body cavities, is not known, in spite of using ozone in the treatment of some diseases. The therapeutic effect of ozone is controversial. Ozone is a strong oxidant, so it's interesting to know if it is able to influence on oxidation of lipoproteins. Lipoproteins modificated in so a way have got some proatherogenic properties. The aim of this publication is to take advantage of NMR spectroscopic technique in the research of ozone Influence on plasma lipoproteins from rabbits with atherosclerosis. ^{1}H-NMR resonance spectra of plasma and plasma lipoproteins from the rabbits with atherosclerosis induced with choiesterol-rich diet, as well as from ozoneted rabbits with atherosclerosis were obtained. Atherosclerosis was induced in 20 New Zealand male rabbits aged about 6 months fed with diet enriched with choiesteroi in the amount of 0.5%. After 3 month feeding with this diet 10 rabbits were treated with medicai ozone in the amount of 0.285 ml/kg body weight, as a mixture O_2/O_3 with ozone concentration of 54 μg/cm^3. Ozone was introduced into marginal vein of ear 2 times a week through 5 weeks. ^{1}H NMR spectra were obtained using Varian VXR-300 spectrometer. In rabbit serum some parameters of lipid metaboiism (concentrations of piasma lipids and fatty acids desaturation) were aiso measured. Lipoproteins were obtained with the precipitation method. The decrease of resonance signals intensity from methyiene protons of lipoproteins of ozonated animais with atherosclerosis was observed in comparison with the group without ozone treatment. On the basis of anaiysis NMR spectra, as well as on the basis of absence statisticaiiy significant changes in lipids concentration and the degree of desaturation of fatty acids between two groups of animals we suppose that the cause of decreased intensity of methyiene signals is probabiy the shortening of carbon chains of fatty acids in lipoproteins.

INTRODUCTION

The harmful effects of ozone inhalations on the living organisms are well known (Banerje & Mudd, 1992; Kennedy, Hatch, Slade & Mason, 1992; Mayer 1992) while the theoretical aspects of ozone use in the therapy are poorly understood (Łaszczyca, Kawka-Serwecińska, Dołeżych, Falkus, Witas, Migula & Madej, 1993). The mechanisms of the action of medical ozone, which is introduced into the arteries, veins or into the body cavities, is not known, in spite of using ozone in the treatment of some diseases. Medical ozone is a mixture of ozone and oxygen. The followers of using medical ozone in the therapy use ozone in the injections, as a medicine in the treatment of many diseases, among others in the patients with lower extremities atherosclerosis (Sroczyński, Antoszewski, Rudzki, Matyszczyk, Krupa, Zbrońska & Skowron, 1992). The important effect of medical ozone is the improvement of serum lipid parameters in the patients with atherosclerosis (Sroczyński, Antoszewski, Rudzki, Matyszczyk & Kuźniewicz, 1990).The therapeutic effect of ozone is controversial, because this method of treatment has also its opponents, who are afraid of side-effects, even distant, of the treatment. These side-effects of ozone therapy are connected with its properties. Ozone is one of the forms of oxygen. Ozone is decomposed into molecular and atomic oxygen (Antoszewski, Lepieszko, Madej & Daniluk, 1993). The effect of free radicals and active forms of oxygen is connected with the pathogenesis of atherosclerosis and other diseases (Esterbauer, Wag & Puhl, 1993). Ozone is a strong oxidant, so it is interesting to know if it is able to influence the oxidation of lipoproteins. Oxidized lipoproteins have some proatherogenic properties (La Ville, Sola, Balanya, Turner & Masana, 1994). Some of these proatherogenic

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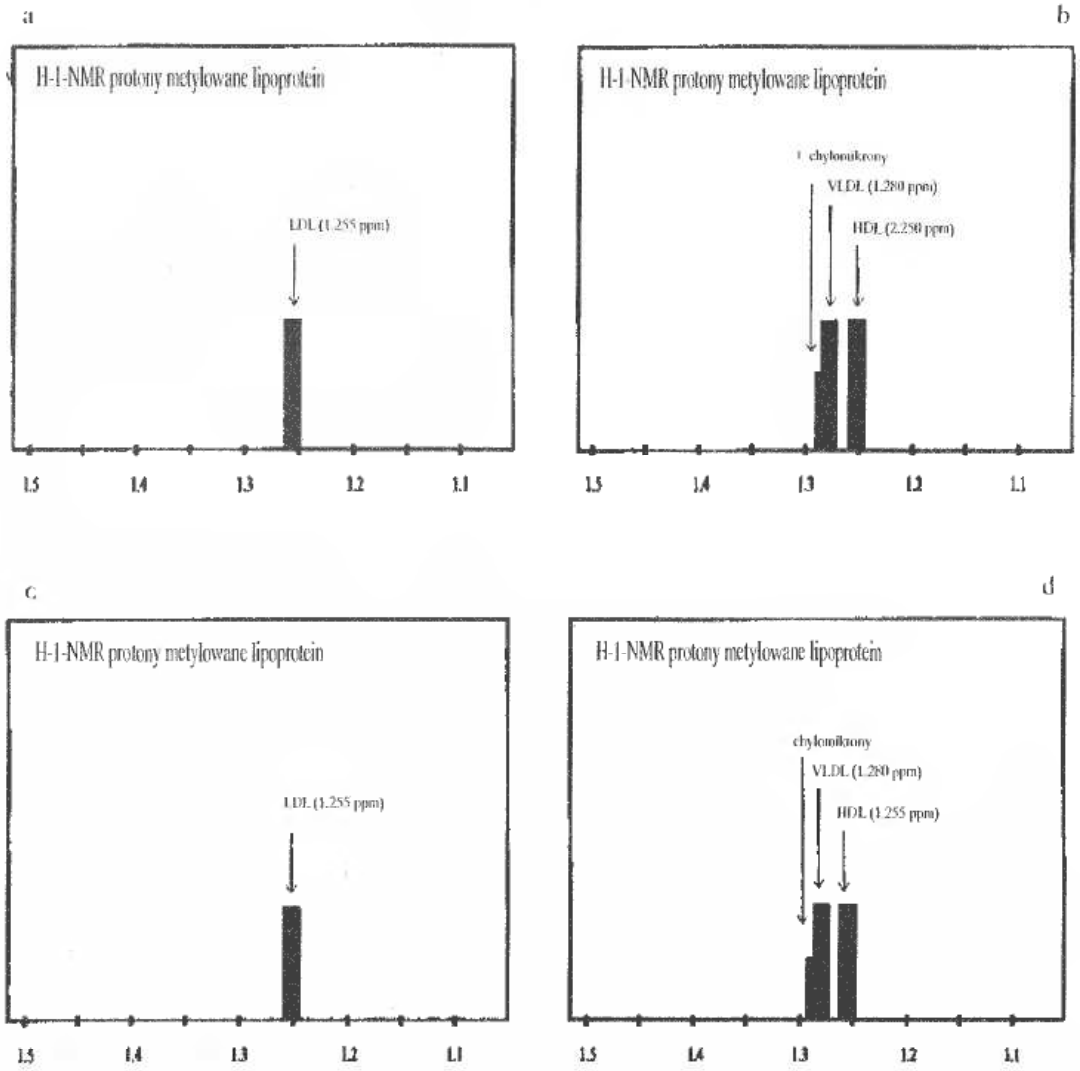

Fig. 1. (a) Integration of methylene protons of ^{1}H NMR spectra of plasma LDL fraction from rabbits with atherosclerosis induced with cholesterol enriched diet. (b) Integration of methylene proton ^{1}H NMR spectra from mixture of plasma HDL and VLDL from rabbits with atherosclerosis induced with cholesterol enriched diet. (c). Integration of methylene proton ^{1}H NMR spectra of plasma HDL fraction of lipoproteins of the rabbits with atherosclerosis induced with cholesterol enriched diet. (d) integration of methylene proton ^{1}H NMR spectra of mixture of plasma non HDL lipoproteins (VLDL and LDL) from rabbits with atherosclerosis induced with cholesterol enriched diet.

properties are: the stimulation of secretion of chemotactic proteins, cytokines and growth factors, the contraction of blood vessels, cytotoxic effects and so on. The aim of this paper is to take advantage of NMR spectroscopic technique to study the ozone influence on plasma lipoproteins from rabbits with atherosclerosis.

METHODS

^{1}H-NMR spectra of plasma from the rabbits with atherosclerosis induced with cholesterol-rich diet, as well as from ozonized rabbits with atherosclerosis were obtained. Atherosclerosis was induced in 20 New Zealand, male rabbits aged about 6 months whose diet was enriched with cholesterol in the amount of 0.5%. 10 rabbits after 3 months on this diet were treated with medical ozone, which was introduced in the amount of 0.285ml/kg body weight, at the concentration of 54 $\mu g/cm^3$, as a mixture O_2/O_3 into marginal vein of the ear, 2 times a week through 5 weeks. Ozone was produced in Biozon Hot apparatus (Vertriebs GmbH). ^{1}H NMR spectra were obtained using a Varian VXR-300 spectrometer operating at 300 MHz, with 10% D_2O, as a signal lock at the temperature of 25°C, diameter of pistel 5 mm. Each

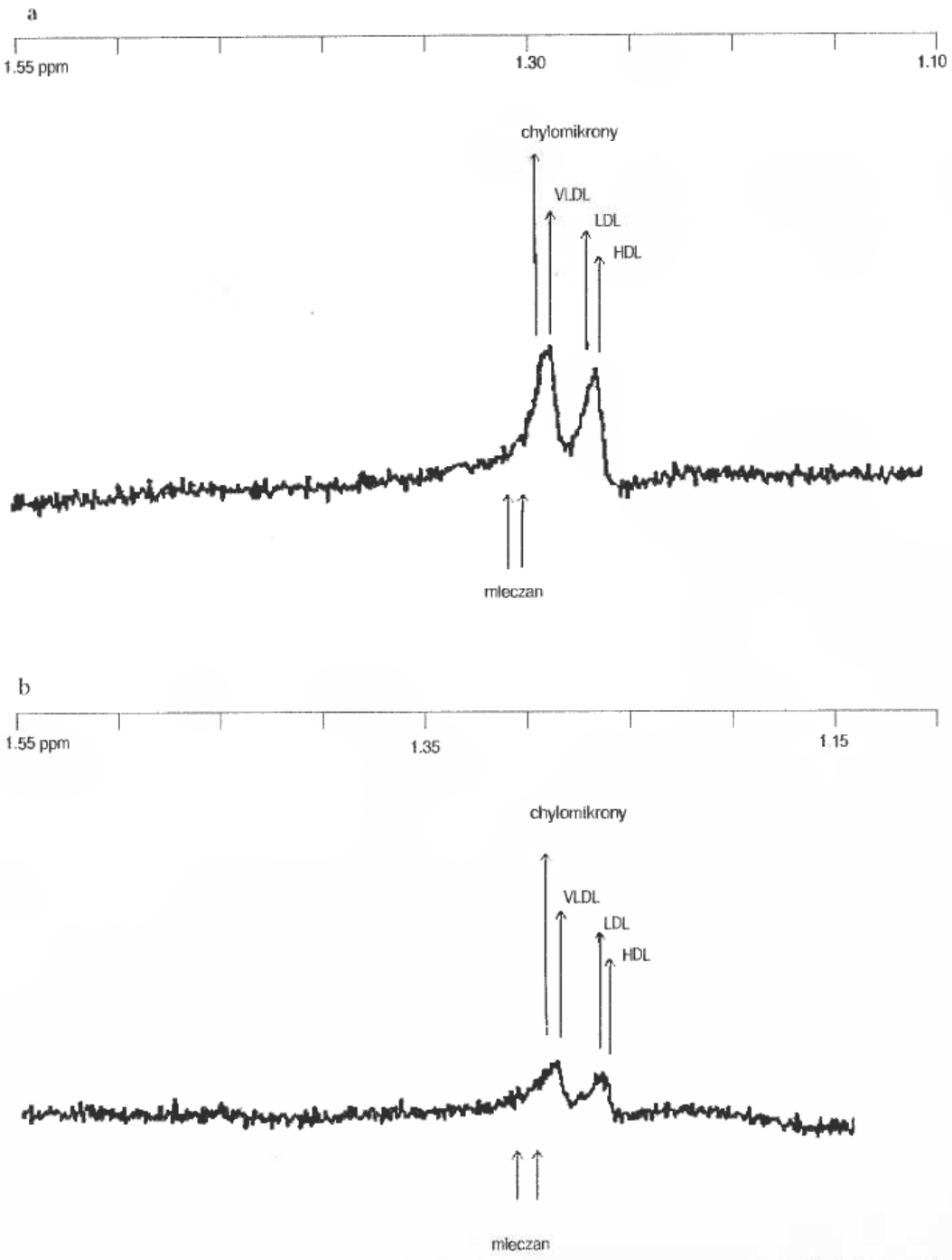

Fig. 2. (a) Histograms of ^{1}H NMR spectra of plasma lipoproteins from rabbits with atherosclerosis induced with cholesterol rich diet. (b). Histograms of ^{1}H NMR spectra of plasma lipoproteins from ozonised rabbits with atherosclerosis induced with cholesterol- rich diet.

spectrum corresponds to 32 free induction decays (FIDs). The large H_2O signal was suppressed by duble resonance method at the H_2O resonance frequency using the decoupler coils.

In rabbit serum some parameters of lipid metabolism, which were essential for discussion were also measured. Total serum cholesterol and cholesterol in the fractions of lipoproteins were estimated with Merck test kits, triglycerides were determined enzymatically with test kit by Boehringer-Mannheim. Total lipids concentration were estimated with a POCh kit. Lipids unsatura-

Tab.1. The influence of ozone on lipid parameters and lipid unsaturation in plasma of rabbits with atherosclerosis.

Parameter measured	Atherogenic group	Atherogenic group with ozone
Total cholesterol (mmol/l)	9.53 ± 5.39	8.57 ± 5.67 p > 0.05
LDL cholesterol (mmol/l)	4.78 ± 1.18	5.28 ± 1.62 p > 0.05
HDL cholesterol (mmol/l)	0.74 ± 0.16	0.68 ± 0.11 p > 0.05
Triglycerides (g/l)	1.1 ± 0.51	0.98 ± 0.57 p > 0.05
Total lipids (g/l)	11.45 ± 3.82	9.86 ± 3.36 p > 0.05
Unsaturation of plasma lipids (g iodine /l)	5.86 ± 1.72	4.25 ± 1.66 p > 0.05
Unsaturation of plasma lipids (g iodine /100g of lipids)	51.18 ± 15.00	43 ± 16.79 p > 0.05

tion was measured through the titration with iodine, using the method of Bauer (1957). The data were analysed using t-Student test.

RESULTS

NMR spectroscopy results are presented in Figs 1 and 2, while the concentrations of the measured lipid parameters are shown in Table 1.

DISCUSSION

The decrease of resonance signal intensity of methylene protons of lipoproteins of ozonized animals with atherosclerosis was observed in comparison with the group without ozone treatment. The reason for this effect may be:

(1) decreased amount of lipoproteins,

(2) increased unsaturation of fatty acids,

(3) the shortening of fatty acids carbon chains.

On the basis of analysis of NMR spectra, as well as on the basis of the absence of statistically significant changes of lipid concentration and the degree of desaturation of fatty acids between two groups of animals we suppose that the cause of decreased intensity of methylene signals is probably the shortening of carbon chains of fatty acids in lipoproteins.

CONCLUSIONS

Ozone decreases the intensity of methylene signals in ^{1}H NMR spectrum of rabbit lipoproteins with atherosclerosis.

A probable reason of decreasing intensifity of methylene signals in spectrum ^{1}H NMR induced with ozone is the modification of hydrocarbon chains of fatty acids, as a result of their shortening.

Ozone does not change, in a statistically significant manner, the measured lipid parameters in rabbits with atherosclerosis.

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Current Topics in Biophysics, vol. 18(2), 216-219

THE EFFECTS OF VITAMINS A, C, E ON THE BALANCE OF XANTHINE OXIDASE (XOD), CATALASE (CT) AND SUPEROXIDE DISMUTASE (SOD) IN NEUTROPHILS (PMN) OF PATIENTS WITH CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD)

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(Accepted 28 December 1994)

A 33–133% increase of CT activity in neutrophils from patients with COPD compared with those from healthly humans was found. The activity of CT decreased after a six week NAC therapy. Then *in vitro* (in tissue culture) investigation of NAC; 50–80% decrease of CT activity in presence of hydralasine and an increase of this activity by ca 30% in presence of vitamin A. Vitamins C and E caused slight increase of the activity CT in some tissue cultures. The presence of vitamins A and C in tissue cultures protected the enzyme activity of CT against influence of NAC. SOD activity remaind unchanged in presence of varied concentrations of vitamins A, C, E. However remarkable fall of SOD (by 70%) was found in NAC presence. The activity of XOD (tested in presence of cytochrome C) increased by 100%, 300% and 500% when NAC, hydralasine or both respectively, were present. XOD activity is not effected by vitamins A nad C.

INTRODUCTION

Free radicals are continuously produced in all aerobic tissues. Neutrophils, monocytes, macrophages, eosinophils and endothelial cells, all generate oxyradicals. It is now generally assumed that oxyradicals play an important role in inflammation-induced tissue injury (Cross, Halliwell, Borish, Pryor, Ames, Saul, McCord & Harman, 1987). The superoxide radical and hydrogen peroxide formed during the respiratory burst of phagocytic cells increasee cytokines, chemotaxis and platelet activation which is essential to sustained tissue injury in chronic inflammatory conditions. Balance disorders of oxidants/antioxidants resulting in formation of oxygen – derived free radicals (RO) are found in many diseases – bronchopulmonary dysplasia, Parkinson's disease, postischemic reperfusion injury, rheumatoid arthritis and other autoimmune diseases (Kehrer, 1993; Bulkley, 1993). Also a big significance is ascribed to RO in pathogenesis of chronic obstructive pulmonary disease (COPD). COPD encloses two pathological entities: chronic bronchitis and emphysemy, often occurring simultaneously. Epidemiologic investigations show that the occurrence of this pathology is ten times more often in cigarette smokers (Ludwig & Hoidal, 1982; McCusker & Hoidal, 1988). This is related to 2–4 times greater number and activity of inflammatory cells in the respiratory tract. The cigarette smoke provides free oxygen redicals and activates the lung macrophages, which produce more endogeneous radicals stimulating the lung tissue destruction. Peripheral blood granulocytes in patients with the mentioned bronchial pathology appear to have increased active oxygen metabolite levels.

The purpose of this work is the investigation of influence of vitamines A, C, E nad NAC (N-acetyl-cysteine) on the activity of xanthine oxidase (XOD), catalase (CT) and superoxide dismutase (SOD) enzyme system in neutrophils (PMN) of patients with COPD. N-acetyl-cysteine is a drug to which a relevant role in COPD treatment is assigned. It is supposed to inhibit the inflammatory reactions through its influence on phagocytic and bacterial function of lung macrophages in case of destructive effect of cigarette smoke. It stabilises the oxidative-antioxidative and protease systems in lung tissue and protects the bronchial epithelium against damaging factors and free radicals.

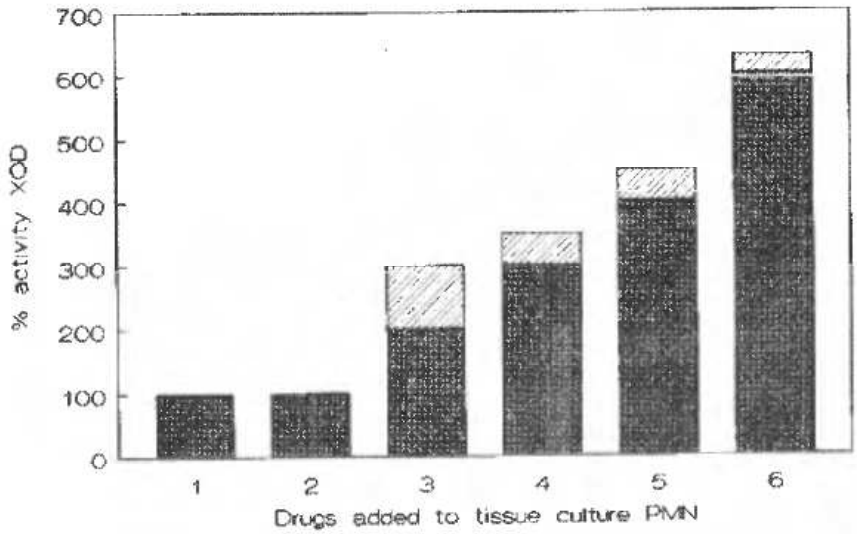

Fig. 1. Effect of NAC, vitamin A and hydralasine on XOD activity. Drugs added to tissue culture PMN (from left to right) 1 – control; 2 – vit. A, 50 U/ml; 3 – NAC, 12 mM; 4 – hydralasine, 0.6 mM; 5 – vit. A, 50 U/ml + hydralasine, 0.6 mM; 6 – NAC, 12 mM + hydralasine, 0.6 mM.

Table 1. Effects of treatment of NAC on CT activity.

Patient's no.	Smoker-s nonsmoker-ns	before treatment U/10^6 cells	after treatment U/10^6 cells
1 A	ns	4.4	–
2 A	ns	4.1	–
3 A	ns	4.1	–
1 B	s	8.9	2.0
2 B	ns	6.9	3.5
3 B	s	6.9	2.8
4 B	ns	4.8	3.5
5 B	ns	4.1	–
6 B	ns	4.2	3.5
7 B	ns	4.2	2.8
8 B	ns	5.5	2.8
9 B	ns	5.5	2.0
10 B	ns	5.5	5.5
11 B	s/ns	6.9	4.8
12 B	s	9.6	6.9
13 B	ns	4.8	2.8
14 B	s	4.1	2.8
15 B	s	8.3	5.5
16 B	s	6.9	5.5

A – healthy nonsmokers
B – patients with COPD

MATERIAL AND METHODS

Neutrophilic leucocytes were isolated by the Boyum method, (Boyum, 1968), from peripheral blood of COPD patients (36 persons) and 8 healthy people (men and women, 40–74 years old).

Tissue culture of neutrophils ($2 \cdot 10^6$) per ml were carried out for 24 hours, at a temperature of 25°C. The cells were lysed by three freeze – thaw cycles.

CT activity was measured in the supernatants by the method Beers and Sizer (1952) with the modification of Freedman *et al.* (Freedman, Anderson & Epstein, 1985). The rate of change per minute in absorbance at 240 nm was recorded. CT activity was calculated using the molar extinction coefficient of 43.6 M^{-1} cm^{-1} for H_2O_2. The phosphate buffer, pH 7, was used.

SOD activity was measured in supernatants, Segura-Aguilar (1993). The amount of H_2O_2 formed by superoxide dismutase was followed by recording the fluorescence of 6,6'-diOH-(1,1'-bipheryl)-3,3'-diacetic acid which is a product of the reaction catalyzed by horseradish peroxidase. The fluorescence were measured in a fluorescence spectrophotometer (Perkin Elmer MPF-3L) using an excitation wavelength of 318 nm and an emission wavelength of 405 nm.

XOD activity was assayed spectrophotometrically by a modification of the method of Salin and McCord (1974). Assays were routinely performed at 25°C in 20 mM sodium carbonate buffer, pH 10, containing 0.1 mM EDTA. Cytochrome c reduction was monitored at 418 nm during transformation of xanthine to uric acid by xanthine oxidase.

RESULTS AND CONCLUSION

CT activity increased from 33% to 133% in neutrophils from patients with COPD compared to

Table 2. Changes of CT activity in tissue culture of PMN (2 x 10^6 cells/ml) of healthy people (A) and of patients with COPD (B).

Culture	Added drugs	A		B	
		U/10^6 cells	% activity	U/10^6 cells	% activity
1	–	4.1	100	8.3	100
2	vit. A	4.6	112	10.8	130
3	vit. C	4.3	105	8.8	107
4	vit. E	4.4	106	8.8	107
5	vit. A, vit. E	4.3	105	8.8	107
6	vit. E, vit. C	4.2	102	8.8	107
7	vit. E, vit. A	4.3	105	8.3	100
8	NAC	0.3	6	1.7	20
9	hydralasine	1.1	27	4.5	52
10	NAC, hydralasine	0.0	0	0.0	0
11	vit. A, hydralasine	0.0	0	0.8	10
12	vit. A, NAC	3.4	83	10.1	122
13	vit. E, NAC	3.1	75	6.3	76
14	vit. C, NAC	3.4	82	6.6	80
15	vit. E, vit. C, NAC	4.2	102	8.8	107

Concentrations of drug added to tissue culture:vit. A– 50 U/ml; vit. C– 0.2 mM; vit. E– 0.3 U/ml; NAC– 12 mM hydralasine – 0.6 mM

Table 3. Changes of SOD activity in tissue culture of PMN (2 x 10^6 cells/ml) of healthy people (A) and of patients with COPD (B).

Culture	Added drugs	A		B	
		U/10^6 cells 10^{-3}	% activity	U/10^6 cells 10^{-3}	% activity
1	–	9.7	100	9.5	100
2	vit. C*	11.5	118	10.4	109
3	vit. C	10.5	108	10.5	110
4	vit. C**	10.1	104	10.8	113
5	vit. C, NAC	3.9	40	4.7	49
6	vit. C, NAC, vit. A	3.9	40	4.7	49
7	vit. A	9.7	100	10.1	106
8	NAC	3.3	34	4.7	49
9	vit. E, NAC	9.7	100	9.5	100

Concentrations of drug added to tissue culture:vit. A– 50 U/ml; vit. C– 0.2 mM, vit. C* – 0.02 mM; vit. C** – 2 mM; vit. E– 0.3 U/ml; NAC– 12 mM

healthy donors (control group) *in vivo*. In this same time we observed a considerably greater CT activity of smoking patients. After treatment with NAC, CT activity was decreased (Tab. 1).

When CT activity was examined *in vitro* in tissue culture, there was an 80% inhibition or a complete loss of activity in the presence of NAC. 50%–80% inactivation of CT was observed after incubation with hydralasine and no activity with both NAC and hydralasine. Addition of vitamin A augmented the CT activity up to 30%, but addition of vitamins C and E only by 7%. Addition of vitamins A, C and E in the presence of NAC protected CT activity (Tab. 2).

Measurement of XOD activity in the presence of cytochrome c a sensitive agent for reducing effect of RO, showed great susceptibility of this enzyme to the influence of additives. When PMN were treated with NAC XOD activity increased by about 100%, after incubation with hydralasine by about 300% and under the influence of both compounds by about 500%. Vitamin A did not change XOD activity (Fig. 1).

We have found that the SOD activity was similar in neutrophils of patients with COPD and of the control group. Vitamins C and A did not effect the SOD activity, although NAC inhibited this activity by 70%. NAC in combination with vitamin E did not change SOD activity (Tab. 3).

These observations indicate that antioxidants, NAC, vitamins A, C and E and hydralasine (RO stimulating factor) used in the experiments modificate enzymatic activity.

In earlier investigations we have found that NAC has a significant positive role in the treatment of COPD. NAC improved respirometric values and also decreased chemiluminescence of PMN (Jankowska, Passowicz-Muszyńska, Mędrala, Banaś & Marcinkowska, 1993). Our studies indicate that CT activity increased in smokers with COPD. Other investigations (McCusker & Hoidal, 1990) have demostrated a similar dependence in alveolar macrophages from cigarette smokers and smoke-exposed hamsters.

Abbrevations used in this paper:

CT – catalase, E.C. 1.11.1.6
SOD – superoxide dismutase, E.C. 1.15.1.1
XOD – xanthine oxidase, E.C. 1.1.3.22
COPD – chronic obstructive pulmonary disease
PMN – polymorphonuclear leukocytes (neutrophils)
RO – oxygen-derived free radicals
NAC – N-acetyl-cysteine

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Current Topics in Biophysics, vol. 18(2), 220-226

PEROXIDATION OF PROTEINS BY FREE RADICALS AND ITS CONSEQUENCES TO LIVING ORGANISMS

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(Accepted 28 December 1994)

Peroxides belong to main modification products of proteins and amino acids by reactive oxygen species. In the absence of other reactants, peroxides are relatively stable, decaying in the time scale of days at room temperature. Ferrous ions and complexes decompose peroxides yielding carbon-centered radicals detectable by spin trapping.

INTRODUCTION

Free radicals are inevitable by-products of biological redox reactions. They are potentially damaging and there are many pathologies in which they may be important (Halliwell, Gutteridge & Cross, 1992). From the biological point of view, there is general agreement that the most significant molecular changes induced by free radicals involve oxidation (Sies, 1991). The Reactive Oxygen Species (ROS) involved in many of these processes have been identified. The principal biological acceptor of free electrons is O_2, which is first converted to superoxide free radical ($O_2^{\cdot-}$). Other oxidants are the alkoxyl ($RO^{\cdot}$) and peroxyl ($ROO^{\cdot}$) free radicals, hydrogen peroxide, high valence forms of transition metals (Bielski, 1990), singlet oxygen $^1O_2$, nitric oxide, semiquinone radicals, and peroxynitrite (Sies, 1993), together with the most reactive of the ROS, the hydroxyl ($HO^{\cdot}$) radical. Reactions of the ROS with biomolecules generally lead to impairment or loss of biological function.

While the most thoroughly investigated biological interactions of ROS are with lipids, in this report we are concerned with the oxidation of proteins. It is well known that the structure and functions of proteins exposed to free radicals are altered. Depending on the free radical involved, the nature of the protein and conditions of the interaction, protein molecules can undergo scission and cross-linking, destruction of amino acids, increase in susceptibility to proteolysis and heat denaturation, and loss of biological function (Wolff & Dean, 1986; Vince & Dean, 1987; Oliver, 1987; Pryor, Dolley & Church, 1984; Richards, Jessup & Dean, 1988; Dean, Nick & Schnebli, 1989; Stadtman, 1993).

There is evidence that such processes can occur *in vivo*, because damage typical of oxidation was found in proteins from cells and tissues exposed to ROS. Thus, characteristic alterations were found in proteins from erythrocytes (Schuurhuis, Hommes, Bos, Molehar & Konings, 1984, Solar, Dulitzky & Shaklai, 1990), chloroplasts (Kyle, Ohad & Arntzen, 1984), mitochondria (Dean & Pollak, 1983), neutrophils (Ashraf, Rokuta, Johnston & Thomas, 1990), plasma (Wayner, Burton, Ingold, Barclay & Locke, 1987), eye lens proteins (Guptasarma & Balasubramanian, 1992), rat brain (Mickel, Oliver & Starke-Reed, 1990), monkey lung fluid (Lenz, Maier & Krombach, 1990), in atherogenic low density lipoprotein (Esterbauer, Gebicki, Puhl & Jurgens, 1992) and in immunoglobin in diabetes and in rheumatic conditions (Jones & Lunec, 1987). However, the biological significance of such observations is difficult to assess. On the one hand, any chemical alterations to cell components may affect their normal functions. On the other hand, Halliwell (1988) has suggested that abundant proteins, such as albumins, may be important biological antioxidants counteracting the effects of ROS. According to this theory, the proteins protect more vital cell components by intercepting the ROS, with the damaged protein molecules quickly replaced.

Our recent studies suggest that some of the products of protein modification by ROS are chemically active (Gebicki & Gebicki, 1993). One such derivative is the hydroperoxide group at-

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tached to the oxidized protein, which can inactivate biological antioxidants such as ascorbate or glutathione (Simpson, Narita, Gieseg, Gebicki, Gebicki & Dean, 1992). We now extend these studies by describing the generation of free radicals from the decomposition of protein peroxide groups and comment on the potential physiological significance of such processes *in vivo*.

MATERIALS AND METHODS

All solutions were made up in water purified by reverse osmosis, followed by passage through a 3 stage Millipore filter system with a final 0.2 μm membrane. Amino acids, proteins and the spin labels were obtained from Sigma (St Louis, MO) and all the other analytical grade reagents from Merck or from Ajax Chemicals (both in Sydney, NSW). Irradiations were performed in the University's ^{60}Co source at dose rates between 41 and 63 Gy/min. Peroxides were measured by the iodometric method (Gebicki & Gebicki, 1993). The ESR spectra were recorded on a Varian E4 instrument.

RESULTS

An important factor in the potential physiological activity of amino acids and protein peroxides is

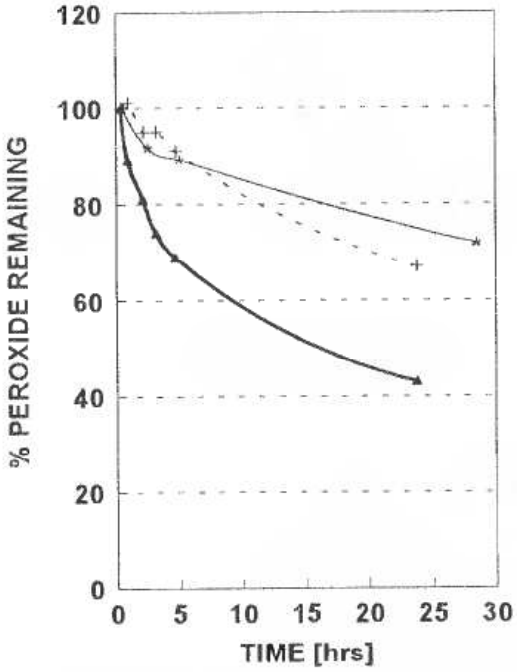

Fig. 1. Spontaneous decay of protein and amino acid peroxides. BSA (5 mg/ml) or valine (20 mM) in 20 mM phosphate buffer pH 7.4 were irradiated with γ rays to doses of 1890 and 1000 Gy, respectively. After treatment with 325 units of catalase/ml, concentration of peroxides was assayed periodically. BSA kept at 20°C, o; valine at 20°C, Σ; BSA at 0°C, +.

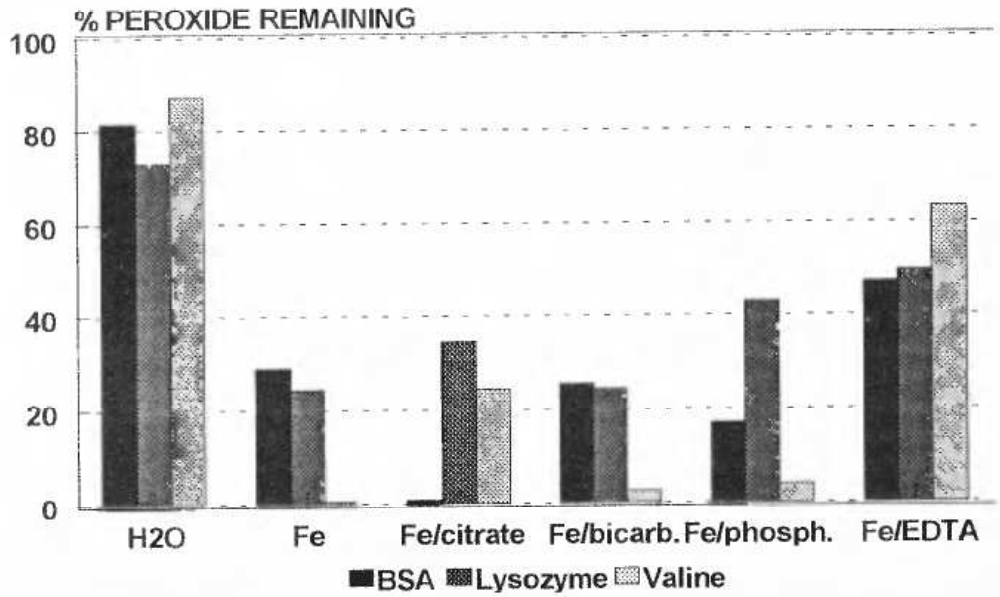

Fig. 2. The effect of chelated and unchelated Fe^{2+} on the rate of decomposition of protein and amino acid peroxides. Valine (20 mM), BSA and lysozyme (10 mg/ml) in 20 mM phosphate buffer pH 7.4 were irradiated with doses of 1000 Gy under an oxygen atmosphere. After treatment with catalase, free or chelated Fe^{2+} were added to final iron concentration of 300 μM. The concentrations of the chelates were 600 μM for EDTA and bicarbonate and 6 mM for citrate. The solutions were incubated for 30 min at 37° and then their peroxide content was measured.

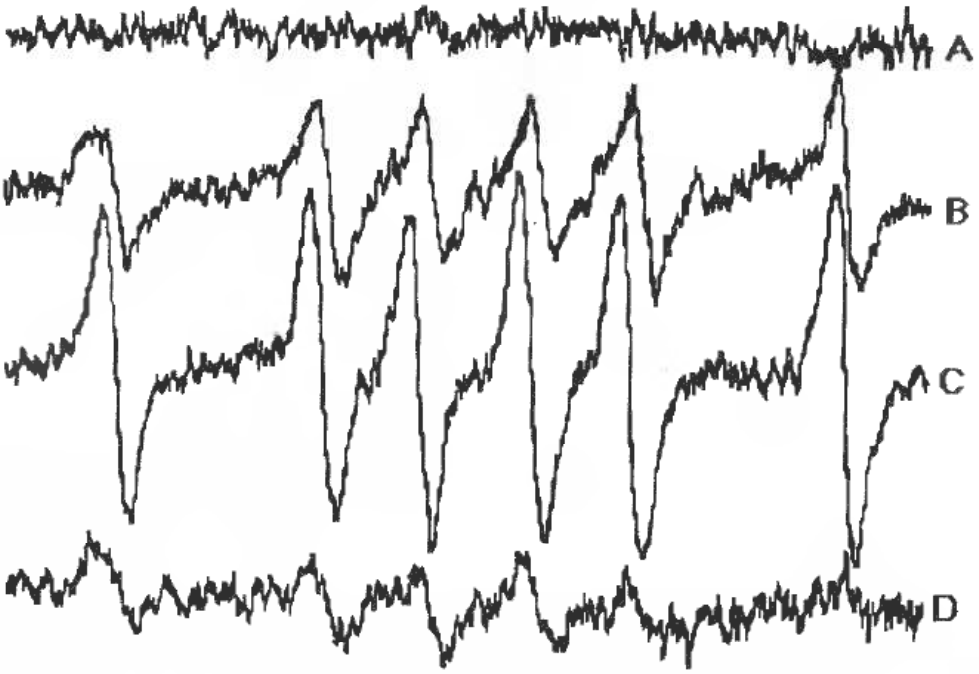

Fig. 3. ESR spectra of oxidized proline. Proline (20 mg/ml) in 0.2 M phosphate buffer pH 7.4 was irradiated at 52 Gy/min and treated with catalase before the addition of Fe^{2+} EDTA in presence of DMPO to final concentrations of 40 μM and 150 mM respectively. The spectra were recorded immediately. A – unirradiated proline; B – proline irradiated with 1040 Gy of γ rays; C – proline irradiated with 6240 Gy; D – proline irradiated to 6240 Gy and then reduced with $NaBH_4$.

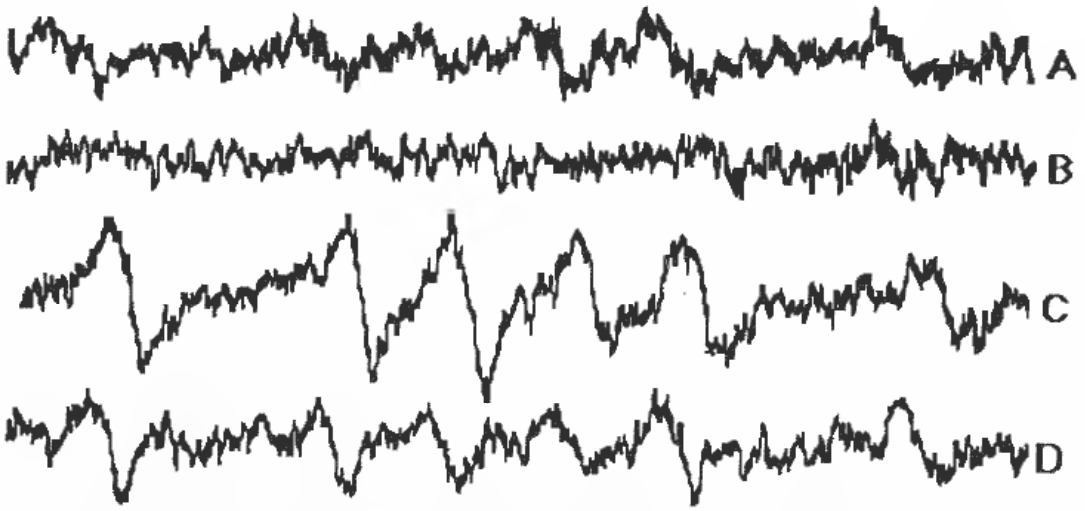

Fig. 4. ESR spectra of irradiated BSA. BSA (20 mg/ml) was irradiated and then treated as the proline samples in Fig. 3. A – unirradiated BSA in presence of Fe^{2+} EDTA and DMPO; B – BSA irradiated with 6240 Gy of γ rays treated with DMPO in absence of Fe; C – BSA treated as in B but with Fe^{2+} EDTA present; D – sample C after reduction with $NaBH_4$.

the stability of the hydroperoxide group. The results shown in Fig. 1 compare the spontaneous decay rates of BSA (bovine serum albumin) and valine peroxides. The peroxides were generated by irradiation of aqueous solutions containing 5 mg/ml of BSA or 20 mM valine, buffered with 20 mM phosphate, pH 7.4. The total γ-ray energy doses were 1890 Gy for the protein and 1000 Gy for the amino acid. The peroxide concentrations were measured by the tri-iodide assay on solutions kept for the indicated times. The graphs demonstrate that, although there was a time-dependent loss of the peroxide groups from both oxidized

molecules, their persistence under the conditions employed was quite pronounced. Protein peroxides decayed faster than amino acid peroxides, but even the former had a half-life of about 36 hrs at 0°C. Another feature of this unassisted decay of BSA peroxides was the stability of some of the peroxide groups; even after 8 days, about 30% of the original peroxides were still present on the protein. While the peroxide decay is speeded up at elevated temperatures, we found that even at 90° it took about 2 hrs to decompose most of the peroxide groups on oxidized BSA. Amino acid peroxides were even less extensively decomposed under such harsh conditions.

The long lifetime of protein peroxides suggested that they could be a source of free radicals and other reactive oxygen species when exposed to agents able to assist their decomposition. Fig. 2 summarises the measurements of the fraction of amino acid and protein peroxides remaining after exposure to free or chelated iron for 30 min at 37°. In all cases the initial peroxide concentrations were comparable, at near 200 μM. We paid careful attention to prevention of the oxidation of the Fe^{2+} before contact with the peroxides. The iron solutions were made up from ferrous ammonium sulphate in water acidified to below pH 2 with sulphuric acid. These solutions were stable for several days. Solutions of chelated Fe^{2+} were prepared by mixing the required amounts of the solutions of the metal and chelate shortly before use.

There were considerable differences in the ability of different reagents to accelerate peroxide decomposition, with no particular obvious pattern. The least reactive with all peroxides was the EDTA chelate. In the 30 min exposure, BSA peroxides were completely decomposed only by the citrate complex, while the valine peroxides were effectively removed by Fe^{2+} and by all complexes tested, except the EDTA. None of the conditions employed led to the loss of more than 25% of the lysozyme peroxides present originally. It seems likely that these results reflect the operation of several factors which play a part in the catalysis of peroxide decomposition by Fe^{2+}. These are likely to include the stability of the Fe^{2+} in solution in a redox-active form and the accessibility of the peroxide group to the catalyst. In the case of the proteins, there may also be some site-specific binding of the Fe^{2+} or its complexes which could affect the peroxide decomposition. Overall, however, it is clear that exposure to Fe^{2+} led in all cases to accelerated peroxide decomposition.

The potential physiological significance of the spontaneous or metal-assisted decomposition is likely to depend primarily on the nature and amounts of the products of this process. Some of these were studied by treatment of peroxidized amino acids and proteins with the slowly-reacting Fe-EDTA complex in the presence of nitroxide spin traps, followed by the recording of any resultant ESR spectra. The results shown in Figs 3-5 demonstrate that in the presence of the spin trap DMPO (5,5-dimethyl-1-pyrroline N-oxide) we could detect the formation of free radicals. The

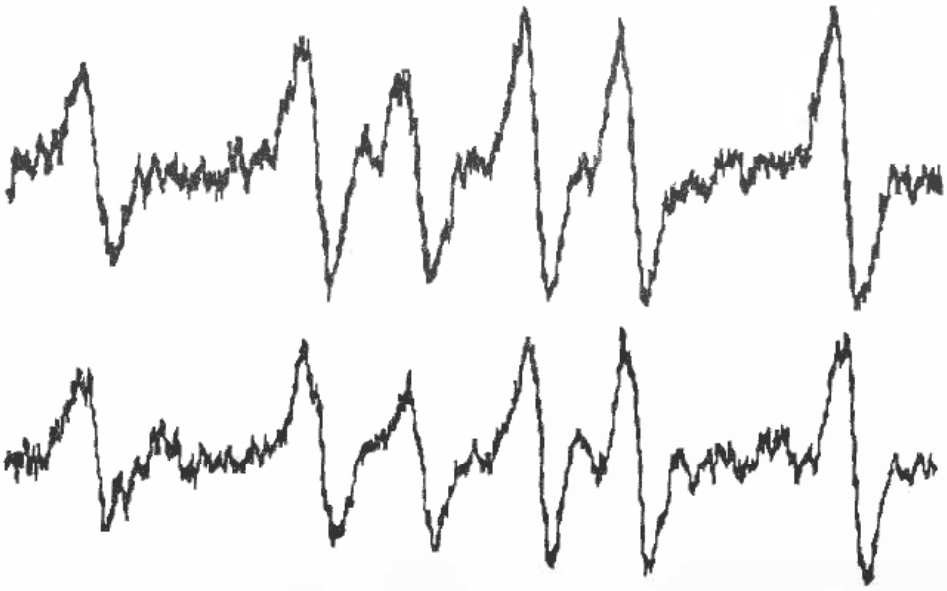

Fig. 5. Comparison of ESR signals generated from cumene hydroperoxide (top spectrum) or from lysine oxidized by γ irradiation. Both compounds were treated with Fe^{2+} EDTA in presence of DMPO.

spectra were recorded at room temperature after rapid mixing of solutions containing the radical-generating amino acid or protein peroxides together with DMPO, and the solution containing free or chelated Fe^{2+}. The strongest signals were recorded with amino acids peroxidized by irradiation and treated with Fe-EDTA in presence of DMPO. A representative example is shown in Fig. 3. Unoxidized proline treated with Fe-EDTA in presence of the spin trap gave no ESR signal. Increasing doses of radiation produced increasingly prominent signals. When the proline peroxide solutions were heated for 3 hr at 90°, most of the signal disappeared. Both the peroxidized BSA and lysozyme gave rise to similar signals after treatment with Fe-EDTA, but their intensity was much lower than for the amino acids known to be susceptible to peroxidation. For BSA, a small signal was recorded with the native protein in presence of DMPO and Fe-EDTA (Fig. 4). This was probably due to the metal-chelating properties of this protein. When the BSA was oxidized by 2 hr exposure to γ-radiation, no ESR signal was observed with DMPO unless Fe^{2+} was added. Further evidence that the spectra were only produced by decomposition of existing peroxide groups and the trapping of the free radicals generated by DMPO was obtained by reduction of the BSA peroxides with sodium borohydride (Fig. 4). Only a small signal was produced in presence of DMPO and Fe-EDTA. We tested a range of amino acids for their ability to give rise to free radicals after irradiation. Pro, lys, val, leu and ile gave strong signals on decomposition of their peroxides by Fe^{2+} in presence of DMPO. Much weaker signals were recorded with glu and gln. No signals were observed with oxidized cys, ser, gly, tyr, thr, arg, asp, asn, met, his, phe or ala. The main components of these and all other spectra consisted of two overlapping triplets of equal intensities, with splitting constants measured as about a^N=16 G and a_β^H = 24 G. Closely similar ESR spectra were recorded by spin trapping of radicals produced during iron-induced decomposition of cumene hydroperoxide (Fig. 5) and all the other amino acid or protein peroxides (here shown for lysine peroxide). These observations are consistent with the formation of free radicals by the metal-assisted decomposition of the amino acid and protein peroxides, with final trapping of carbon-centred radicals by the DMPO.

DISCUSSION

Discussion of the potential significance of protein oxidaton to living organisms in current literature has mainly concentrated on two aspects of this process: the major one, suggesting that the products of the oxidation can accumulate and impair the normal functions of cells (Stadtman, 1990), and a less extensive speculation on the role proteins may play in protecting cells from damage by ROS (Halliwell, 1988). The possibility that proteins may themselves be a source of free radicals has only arisen after the discovery of the generation of potentially reactive oxidizing and reducing groups on the molecules affected by ROS (Simpson *et al.*, 1992; Gebicki & Gebicki, 1993).

In this study, we have confirmed recent observation of formation of free radicals from proteins carrying the peroxide groups made by M. J. Davies (private communication). In his work, most of the radicals were identified by careful analysis of the ESR spectra obtained by the capture of reactive species with several spin trapping agents. They included alkoxyl, superoxide, carbon dioxyl, and some unidentified free radicals, mainly carbon-centred. The radicals were derived from protein and amino acid peroxides exposed to chelated iron. Our results also show that the prerequisite for the generation of these radicals is the presence of peroxide groups on the protein or amino acid. We have previously shown that both BSA and lysozyme are susceptible to peroxidation by exposure to free radicals in presence of oxygen, while of the 20 common amino acids only 6 form peroxides in comparable yields (Gebicki & Gebicki, 1993). The ESR results obtained in this study are in satisfying agreement with the earlier work. The same amino acids which form peroxides gave rise to free radicals on their decomposition, and there was a correspondence between the somewhat lower peroxide and free radical yields in the case of glutamic acid.

At this point, we have no indication whether the processes of protein peroxidation and peroxide decomposition occur *in vivo*. As in the case of most studies of the biology of free radicals, existing technology only allows the conclusions that ROS form in living organisms and that their formation leads to physiological consequences (Halliwell & Gutteridge, 1989). Reactions which may involve such reactive species *in vivo* are generally not capable of direct study, so that mechanistic interpretations must be derived from essentially test-tube studies of reactions under biologically plausible conditions. One of the major difficulties in accounting for the many well-documented biological effects of ROS is the very limited radius of effectiveness imposed by the short lifetime of the most physiologically important radicals (Sies, 1993). Of these, only the NO survives long enough to traverse a blood vessel (Beckman, Chen, Ishiropoulos,

Zu, Conger & Halsey, 1993), while the $HO^{\cdot}$, which is generally believed to be the main radical agent of biological change, reacts virtually at its site of formation. Thus, if no carriers of the chemical energy of the free radicals existed, the nature of the biological damage induced by most radicals would be determined by the point at which they are produced. There are many indications that this is not likely.

Our studies suggest that proteins can trap part of the energy of the ROS in the form of peroxide groups (Simpson *et al.*, 1992). The probability of the interaction of free radicals released in a living organism with proteins is higher than for any other molecule, on a mass basis. The protein peroxides produced in some of these reactions have a very long lifetime compared to the radicals causing their formation, at least *in vitro* (Fig. 1). The processes causing the slow spontaneous decay of the peroxide groups are unknown, but are likely to involve reactions with components typically present in proteins. These may be other amino acids or species present as impurities or cofactors. We know that the reaction between protein peroxide groups and transition metals is fast, and it is difficult to ensure complete abence of metals from molecules such as BSA. The much slower decay of valine peroxide (Fig. 1) supports this suggestion. The likely lifetimes of protein peroxides *in vivo* are unknown. So far there is only one preliminary observation of their accelerated removal by rat liver extracts (Dean, Gebicki, Gieseg, Grant & Simpson, 1992). Diffusion of peroxidized proteins to distant sites appears therefore feasible. This would allow them, on the one hand, to lower the antioxidant potential of tissues by removing intracellular (glutathione) or extracellular (ascorbate) antioxidants, and, on the other hand, to initiate further chemistry though the production of new radical species. We are currently engaged is studies of the changes which these radicals may be capable of inducing in other biomolecules.

Acknowledgements

This work was supported by grants from the Australian Research Council, the Royal Australian College of Physicians and Macquarie University. SG is the holder of an Australian Postgraduate Award.

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Current Topics in Biophysics, vol. 18(2), 227-231

DAMAGE TO HUMAN ERYTHROCYTES BY γ-RADIATION UNDER AIR, NITROUS OXIDE AND ARGON

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(Accepted 28 December 1994)

Erythrocyte suspensions in isotonic saline (2% hematocrit) were irradiated from a ^{60}Co source under air and under anoxic conditions that is under the atmosphere of N_2O or argon. Erythrocyte damage was estimated on the basis of an analysis of dose-response curves carried out for the the hemolysis of erythrocytes as well as for changes in the shape and size of cells (prehemolytic changes). Erythrocyte radiosensitivity defined on the basis of D_{37} dose for hemolysis is 2.1 times higher under air than under argon, and 1.7 times higher under air than under N_2O. However, radiosensitivity determined on the basis of prehemolytic changes is 4.3 times higher under air than under anaerobic conditions. The results obtained are discused from the point of view of contribution of water radicals to the radiation damage to human erythrocytes and indicate that molecular oxygen enhances the destruction of erythrocytes initiated by hydroxyl radicals.

INTRODUCTION

The action of ionizing radiation on living systems is via ionizing and excitation of atoms and molecules within important cellular components. Radiation produces ionizations and excitations at random, so that in a complex system such as living matter, most abundant molecules are those most likely to become ionized. Beause mammalian cells are approximately 70% water, most of the energy will be taken up by water molecules. Highly reactive species are formed by water radiolysis (e^-_{aq}, $^{\cdot}OH$, H, H_2O_2). They are able to react with important cellular molecules and cause radiobiological damage. This process is known as indirect action. In contrast, the direct action of radiation involves simple interaction between the ionizing radiation and target molecules. However, it is probable that most radiobiological damage is a consequence of indirect action (Coggle, 1983; Kiefer, 1990; Lea, 1946; Michaels & Hunt, 1978).

The aim of the present paper was to study the effect of primary water radicals and oxygen on the damage of erythrocytes leading to their hemolysis. In order to study these effects the radiolysis of erythrocytes was carried out under air, nitrous oxide and argon.

MATERIALS AND METHODS

Materials

Human erythrocytes were obtained from the blood of healthy donors anticoagulated with ACD (trisodium citrate and glucose).

Erythrocytes were separated from blood plasma and leucocytes by centrifugation at 4°C at 2000 g for 10 min. They were futher washed three times with isotonic saline, resuspended in saline to obtain a hematocrit of 2% and irradiated.

Irradiation conditions

Erythrocyte suspensions (2% hematocrit) were irradiated from a ^{60}Co-γ-radiation source, at room temperature. The dose-rate estimated with a modified Fricke dosimeter was 4kGy h^{-1}. The suspensions were irradiated under air and under anoxic conditions that is under the atmosphere of N_2O or argon. During irradiation the erythrocyte suspensions were stirred with a magnetic bar.

Flow cytometry

0.5 ml samples of density of about 10^6 cells/ml were analysed using an ARGUS flow cytometer (Scatron). The results were analysed by an IBM computer with the basic company program of the ARGUS cytometer.

The measurements included:

low angle light scattering (LS-1);

light scattering at an angle of 90° (LS-2).

The percentage of altered cells was read from histograms and also the profiles of histograms were analysed (Steen, 1983).

Determination of hemolysis

The percentage of hemolysis was determined by the measurement of hemoglobin released from cell relative to the total cellular hemoglobin content.

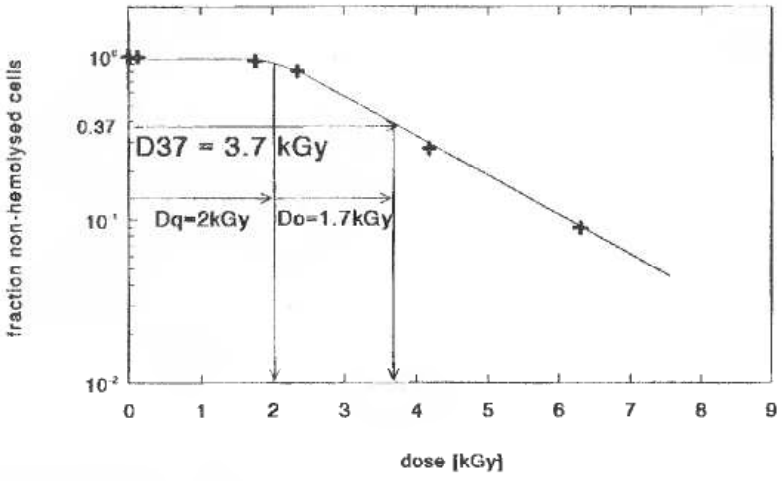

Fig. 1. Dose-response curve for hemolysis of human erythrocytes irradiated under air. D_q – the quasi threshold dose, which is the intercept of the back-extrapolated terminal straight of the curve on the 100% non-hemolysed cells axis; D_0 – the dose required to reduce non-hemolysed cells to a fraction of 0.37, along exponential part of curve; $D_{37} = D_q + D_0$.

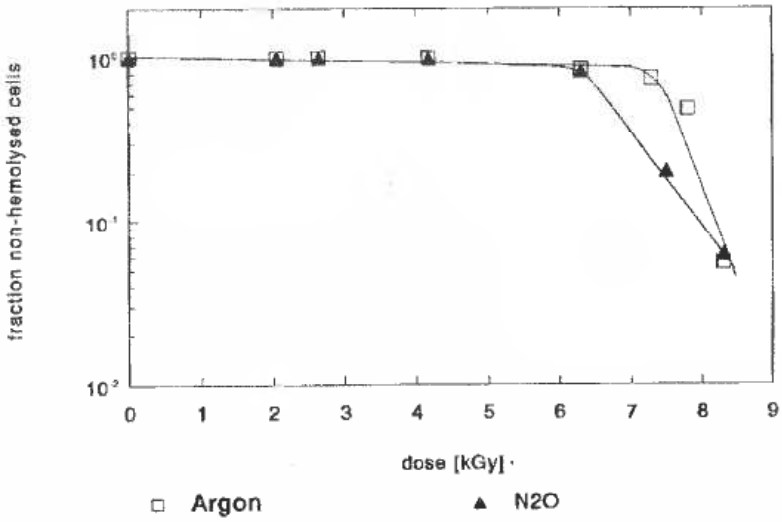

Fig. 2. Dose-reponse curves for hemolysis of human erythrocytes irradiated under N_2O and argon.

Hemoglobin was estimated on the basis of absorbance at 630 nm after oxidation of iron with $K_3[Fe(CN)_6]$.

RESULTS

Radiation-induced hemolysis of erythrocytes

The final radiation damage effect of erythrocytes is hemolysis. This appears to be a classical example of "interphase death" which is rather uncommon in actively proliferating cells. Figures 1 and 2 show the dose-response curves for the hemolysis of human erythrocytes irradiated under various conditions. The shape of the curves is similar to that found for radiation-induced loss of reproductive capacity in mammalian cells. They have a "shoulder" at low doses and became exponential only at higher doses. The shoulders of the curves are described by the dose D_q – the "quasi threshold" dose. This is an indication of the ability to accumulate substantial damage before lysis and to release of hemoglobin. This is often referred to as a sublethal damage. At the doses greater than D_q, the log of non-hemolysed erythrocytes decreases linearly, indicative of pseudo-first-order kinetics. The slope of this region is defined as the rate constant of hemolysis (k) which is commonly indicated by the dose termed D_0" ($D_0 = 1/k$).

Tab. 1 summarizes the parameters of the dose-response curves for hemolysis of human erythrocytes irradiated under the various conditions employed. In all cases there are obvious differences in the dose D_q, D_0 and D_{37}. The lowest dose value D_q was found for preparations irradiated under air. However under anoxic conditions the dose D_q increased 3 fold in the case of N_2O and 3.6 fold in the case of argon. The high values of D_q doses were accompanied by the low values of D_0 doses (high k value). So hemolysis takes place most rapidly in suspensions of erythrocytes irradiated under argon $k = 2.5$ kGy^{-1}. This process takes

Table 1. Parameters of dose-response curves for hemolysis of erythrocytes irradiated under different conditions.

Irradiation conditions	D_q [kGy]	D_0 [kGy]	D_{37} [kGy]
Air	2.0	1.7	3.7
N_2O	6.1	0.8	6.9
Argon	7.3	0.4	7.7

Table 2. Parameters of dose-response curves for size of erythrocytes irradiated under different conditions.

Irradiation conditions	D_q [kGy]	D_0 [kGy	D_{37} [kGy]
Air	0.8	0.23	1.03
N_2O	3.4	0.9	4.5
Argon	3.4	0.9	4.5

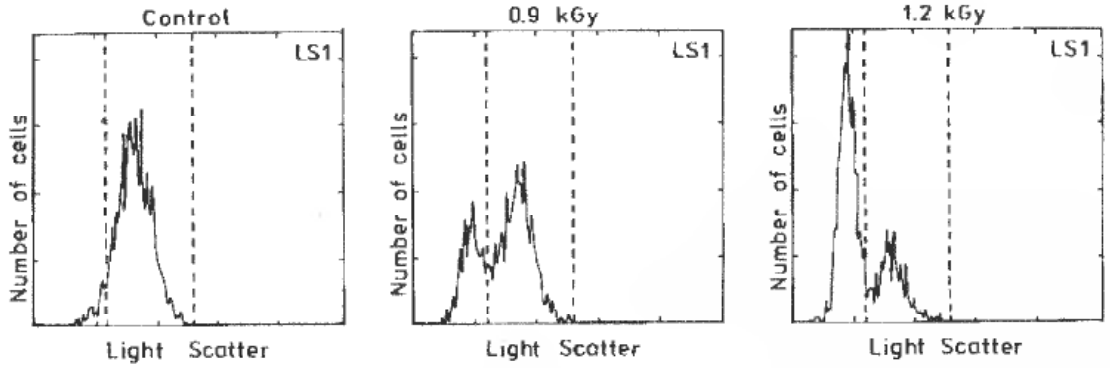

Fig. 3. Flow cytometric analysis of the control erythrocytes and samples irradiated under air with the following doses 0.9 kGy, 1.2 kGy.

place two times more slowly under N_2O (k = 1.25 kGy^{-1}). However when erythrocytes are irradiated under air the hemolysis is the slowest (k = 0.56 kGy^{-1}). The obtained results indicate the high cooperativity of the hemolysis of erythrocytes irradiated under anoxic conditions.

The coefficient of the oxygen effect which is called "oxygen enhancement ratio" (OER) calculated on the basis value of D_{37}

$$\left(\mathrm{OER} = \frac{D_{37} \text{ in the presence argon}}{D_{37} \text{ in the presence } O_2}\right)$$

is equal to 2.1. This indicates that erythrocytes irradiated in the presence of oxygen are damaged more heavily than under anoxic conditions.

Radiation-induced size changes of erythrocytes

Light scattering by human erythrocytes irradiated under air, N_2O and argon with the dose lower than D_q (non-hemolysing doses during irradiation) was measured by flow cytometry. Red cell features that contribute to light scattering are the cell size, shape and structure of the cell membrane. The analysis of the histograms indicated a decrease in cell sizes and a transition to a more spherical shape. Typical histograms obtained on the basis of light collected in the forward direction for control cells (unchanged) and cells irradiated under air are presented in Fig. 3. The peak corresponding to the cells whose shapes and sizes were unchanged became smaller with increasing doses. Simultaneously there was on increase of peak containing cells with changed sizes. If the fraction of the unchanged cells was plotted versus dose in a half logarithmic scale, typical "survival curves" were obtained with a "shoulder" and an exponential part (Fig. 4).

The parameters characterizing these curves are given in Tab. 2. The threshold dose D_q and dose D_0 had the lowest values for erythrocytes irradiated under air. Under these conditions size changes of erythrocytes appear after a dose of 0.8 kGy. Increase in the fraction of changed cells took place at a constant rate of 4.3 kGy^{-1} (D_0 = 0.23 kGy) and at the dose of 2 kGy this fraction was about 100%. It should be pointed out that 2 kGy is the dose D_q after which hemolysis begins. In the case of irradiation of erythrocytes under N_2O and argon no differences were observed in the course of the dose-response curves. The D_q dose determined for erythrocytes irradiated under anaerobic conditions was 4.25 times higher than D_q deter-

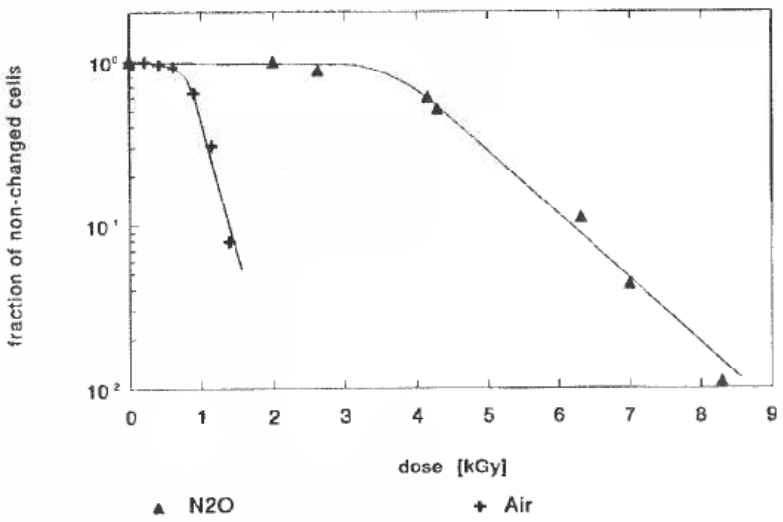

Fig. 4. Dose-response curves for size changes of human erythrocytes irradiated under air and N_2O.

mined for erythrocytes irradiated under air. However, the rate constant for the disapperance of unchanged cells was equal to 1.1 kGy^{-1} (D_0 = 0.9 kGy). This means that changes in the size of cells take place 4 times more slowly under anoxic conditions than in the presence of oxygen. The OER calculated on the basis of dose D_{37} indicates an about 4 fold higher sensitivity of erythrocytes to changes in size and shape during irradiation in the presence of oxygen, in comparison with anaerobic conditions.

It is noteworthy that the above changes in the sizes of erythrocytes take place in the range of doses corresponding to the length of the shoulder of dose-response curves for hemolysis. So they can be described as sublethal prehemolytic damage to erythrocytes.

DISCUSSION

The current study was concerned with the kinetics of hemolysis of erythrocytes as well as the kinetics of prehemolytic changes which were initiated mainly by water radicals. During the irradiation of erythrocytes under argon the principal water radical species initiating erythrocyte damage are: hydroxyl radicals $^{\cdot}OH$ (G_{OH} = 2.7), hydrated electrons e^-_{aq} ($G_{e^-_{aq}}$ = 2.6) and hydrogen atoms H (G_H = 0.6) (Buxton, Greenstock, Helman & Ross, 1988). When the radiolysis of erythrocytes takes place under N_2O the e^-_{aq} are scavenged and converted into $^{\cdot}OH$ radicals. Under these conditions the radiation-chemical yield of $^{\cdot}OH$ is about 5.3. In view of the low radiation-chemical yield of H (G_H = 0.6), $^{\cdot}OH$ radicals seem to play a dominant role in the damaging of erythrocytes.

On the basis of marked parameters we can conclude that the radiosensitivity of erythrocytes in an atmosphere of N_2O is close to that of argon. The parameters describing the hemolytic curves differ very little (Fig. 2, Tab. 1). Prehemolytic changes in the shape and size of erythrocytes show identical curves. Considering the radiation-chemical yield of water radicals generated in the presence of N_2O and argon one can say that e^-_{aq} contribute a great deal to erythrocyte membrane damage. Since e^-_{aq} are characterized by a low reactivity with lipids (Wolff, Garner & Dean, 1986) we can suggest that they mainly attack proteins (e^-_{aq} react with proteins with rate constants of $\sim 10^{10}$ $dm^3 \cdot mol^{-1} \cdot s^{-1}$).

On the other hand the presence of the reducing products of radiolysis of water (e^-_{aq}, H) and oxidizing ($^{\cdot}OH$) in more or less equal proportion during irradiation under argon can lead to processes of molecules reconstruction ($R^{\cdot +} + e^-_{aq} \rightarrow R$).

However the results obtained do not indicate that this was the dominant process. Under an oxic (air) atmosphere e^-_{aq} and H react with oxygen (are scavenged by oxygen: $e^-_{aq} + O_2 \rightarrow O_2^{\dot{-}}$; $H + O_2 \rightarrow HO_2^{\cdot}$, $G_{O_2^{\dot{-}}} = 3.3$). In such a system due to the low $O_2^{\dot{-}}$ reactivity, damage will be initiated principally through $^{\cdot}OH$ radicals ($G_{^{\cdot}OH}$ = 2.7). The studies on the influence of $^{\cdot}OH$ scavengers on the level of erythrocyte damage (Bartosz & Leyko, 1981; Miller & Raleigh, 1983) are proof of the dominating role of $^{\cdot}OH$ radicals in radiation damage to erytrocyte.

In spite of the two fold lower radiation yield of $^{\cdot}OH$ radicals under air in comparison to N_2O the radiosensitivity of erythrocytes under air determined on the basis of hemolysis

$$\left(\frac{D_{37} \text{ under } N_2O}{D_{37} \text{ under air}}\right)$$

is about 1.7 fold greater that under N_2O. Radiosensitivity of erythrocytes estimated on the basis of prehemolytic changes indicates that the shape and size of cells is a more sensitive parameter than hemolysis. And so radiosensitivity of erythrocytes estimated on the basis of this parameter is about 4 times greater under oxic than anaerobic conditions.

The results indicate that $^{\cdot}OH$ radicals are far less toxic in the absence of oxygen in other words molecular oxygen enhances the destruction of erythrocytes initiated by hydroxyl radicals.

Acknowledgments

The authors gratefully acknowledge the continuous support ans interest in this work by Professor Dr W. Leyko.

This work was supported in part by Foundation for Radiation Research (Poland).

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Current Topics in Biophysics, vol. 18(2), 232-237

MEMBRANE TRANSPORT OF GLUTATHIONE CONJUGATES

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(Accepted 28 December 1994)

Products of S-conjugation and of oxidation of glutathione (glutathione S-conjugates and glutathione disulfide, respectively) are actively exported from cells by protein(s) called glutathione S-conjugate pump. The identity and mechanism of action of the pump still remain elusive.

The tripeptide glutathione (γ-glutamyl-cysteinyl-glycine; GSH) performs three main functions in the cell: reduction of peroxides (catalysed by glutathione peroxidase and lipid hydroperoxide glutathione peroxidase), inactivation of reactive electrophiles of endo- and exogenous origin (catalysed by glutathione S-transferases), and maintenance of a proper oxidation state of protein -SH groups (Deneke & Fanburg, 1989; Gilliland, 1993; Meister, 1981; 1988; Sies, 1989; Sunde, Dyer, Moran, Evenson & Sugimoto, 1993). Reduction of peroxides and protein disulfide groups generates glutathione disulfide (GSSG) while reactions of glutathione S-transferases yield glutathione S-conjugates. Cells have the ability of active export of GSSG and glutathione S-conjugates; in the case of glutathione conjugates the extrusion of glutathione conjugates out of the cytoplasm is often referred to as detoxication phase III (Ishikawa, 1992).

First observations of GSSG transport across the erythrocyte membrane came from the laboratory of Beutler. They showed that oxidation of GSH in human erythrocytes (by H_2O_2, methyl phenylazoformate or other oxidants) induces GSSG export from the cells (Srivastava & Beutler, 1969a,b). GSSG is transported even if its concentration outside the cells is higher than inside. GSSG transport is inhibited by F^-; its rate is strongly temperature-dependent and decreases when cellular ATP concentration is lowered. The transport is unidirectional; extracellular GSSG does not enter erythrocytes. On the basis of these observations, active export of GSSG from erythrocytes has been postulated (Beutler, 1983). GSSG export has also been demonstrated in lens (Srivastava & Beutler, 1968) and liver (Sies & de Graf, 1985).

More exact information on the GSSG transport across the erythrocyte membrane could be derived from studies of membrane vesicles. GSSG is accumulated in inside-out membrane vesicles provided Mg^{2+} and ATP are present in the medium. Kinetic analysis of the concentration dependence of GSSG transport into inside-out vesicles of human (Akerboom, Bartosz & Sies, 1992; Bartosz, Sies & Akerboom, 1993; Kondo, Dale & Beutler, 1980, 1981) and rat (Heijn, Oude Elfernik & Jansen, 1992) erythrocytes points to the existence of two components of the ATP-dependent GSSG transport of different K_m and V_m values (Fig. 1). The simplest hypothesis to account for these findings implies the existence in the erythrocyte membrane of two various GSSG-transporting proteins (Table 1) of different properties, including sensitivity to -SH reagents (the low-affinity transporter being more sensitive). Both transport components are inhibited by fluoride and vanadate.

The data presented do not allow to determine whether the apparently active GSSG transport is a "primary" active transport or a "secondary active transport" i.e. a passive transport carried out at the expense of an electrochemical gradient of Na^+ or some other cation, or of membrane potential.

If the GSSG transport is a primary active transport, an existence of a GSSG-stimulated ATPase activity can be expected in the plasma membrane. Such an activity has been reported for hepatocyte (Nicotera, Baldi, Svensson, Larsson, Bellomo & Orrenius, 1985a; Nicotera, Moore, Bellomo, Mirabelli & Orrenius, 1985b) and erythrocyte (Kondo, Kawakami, Taniguchi & Beutler, 1987) plasma mebranes. The demonstration and measurement of this activity is not easy since GSSG,

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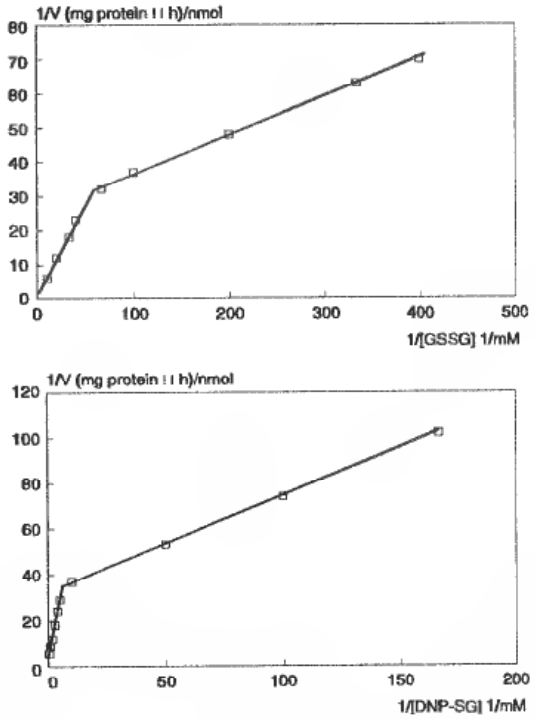

Fig. 1. Lineweaver-Burk plots of the uptake of GSSG and DNP-SG by inside-out vesicles of human erythrocyte membranes. Results of a typical experiment.

while stimulating the specific GSSG-ATPase activity, inhibits other membrane ATPase activities (Kondo *et al.*, 1987).

Kondo *et al.* (1987), using affinity chromatography on S-hexylglutathione-Sepharose column, isolated from human erythrocyte membranes two proteins of GSSG-ATPase activities of different K_m values for GSSG and various sensitivities to SH reagents (Table 2), representing apparently the two active GSSG transporters (Kondo *et al.*, 1987; Kondo, Miyamoto, Gasa, Taniguchi & Kawakami, 1989). The high-affinity GSSG-ATPase has an apparent molecular weight of 150 000 and is composed of two subunits (82 000 and 62 000). This protein was reconstituted in liposomes and shown to transport one GSSG molecule per no more than one molecule of ATP hydrolysed. A similar, if not identical protein was isolated from human liver (Kondo, Miyamoto, Gasa, Taniguchi & Kawakami, 1989).

Export of 2,4-dinitrophenyl-S-glutathione (DNP-SG), the product of conjugation of glutathione with 1-chloro-2,4-dinitrobenzene has been observed in several cell types including erythrocytes (Awasthi, Misra, Rassin & Srivastava, 1983; Board, 1981; Eckert & Eyer, 1986; LaBelle, Singh, Srivastava & Awasthi, 1986a), hepatocytes (Akerboom, Narayanaswami, Kunst & sies, 1991; Kobayashi, Sogame, Hara & Hayashi, 1990; Kunst, Sies & Akerboom, 1983), cardiomyocytes (Ishikawa, 1989; Ishikawa, Kobayashi, Sogame & Hayashi, 1989), K562 erythroid cells (Kondo, Yoshida, Urata, Goto, Gasa & Taniguchi, 1993), neutrophiles (Scott, Matin & Hamilton, 1990) and human colon adenocarcinoma Caco-2 cells, although in the latter case (in contrast to erythrocytes) the transport was sensitive to dissipation of membrane potential (Oude Elferink, Bakker & Jansen, 1993) (Table 2).

In erythrocytes the export rate of DNP-SG is strongly affected by temperature and is diminished upon metabolic depletion (Awasthi *et al.*, 1983; Awasthi, Singh, Wronski, Srivastava & LaBelle, 1989). Accumulation of DNP-SG in inside-out vesicles of human erythrocyte membranes requires ATP and Mg^{2+}. The transport is inhibited by vanadate and fluoride but not by ouabain (inhibitor of $(Na^+,K^+$-ATPase) or EGTA (inhibitor of Ca^{2+}-ATPase). These results suggest that the DNP-SG transport in erythrocytes, like that of GSSG, is a primary active transport (LaBelle *et al.*, 1986a). Two kinetic components of DNP-SG transport across the erythrocyte membrane have been revealed (Akerboom *et al.*, 1992; Bartosz *et al.*, 1993; Ecker & Eyer, 1986) (Fig. 1; Table 3). The high-K_m (low-affinity) component is more sensitive to thiol reagents (N-ethylomaleimide, Ellman

Table 1. Characteristics of two components of GSSG transport across the erythrocyte membrane.

Parameter	High-affinity component	Low-affinity component	Source
K_m(GSSG)	100 μM	7.3 mM	Kondo *et al.*, 1981
	26 μM*	4 mM*	Heijn *et al.*, 1992
	23 μM	5 mM	Akerboom *et al.*, 1992
V_m	20 nmol/(ml vesicles × h)	210 nmol/(ml vesicles × h)	Kondo *et al.*, 1981
	6 nmol/(mg protein × h)*	22 nmol/(mg protein × h)*	Heijn *et al.*, 1992
	6 nmol/(mg protein × h)	66 nmol/(mg protein × h)	Akerboom *et al.*, 1992
K_m(ATP)	0.63 mM	1.25 mM	Kondo *et al.*, 1981
	0.125 mM*	20 μM*	Heijn *et al.*, 1992
	0.13 mM		Akerboom *et al.*, 1992
pH opimum	≤6.5	7.2	Kondo *et al.*, 1981

Human or *rat erythrocytes.

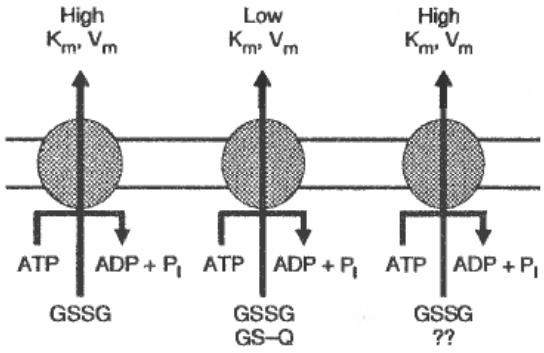

Fig. 2. Postulated relationship between the active transporters of GSSG and DNP-SG in the erythrocyte membrane.

reagent) than that that of low K_m (high affinity) for DNP-SG (Akerboom *et al.*, 1992).

Awasthi *et al.* reported isolation from human erythrocyte membranes (Sharma, Gupta, Singh, Medh, Ahmad, LaBelle & Awasthi, 1990), liver (Awasthi, Singhal, Gupta, Ahmad, Zimniak, Radominska, Lester & Sharma, 1991) and muscles (Saxena, Singhal, Awasthi, Singh, LaBelle, Zimniak & Awasthi, 1992) a protein of molecular weight of 38 000 of a DNP-SG-stimulated ATPase activity. Recently a protein of similar functional properties but of molecular weight of about 90 000 was isolated from rat liver and shown to transport actively DNP-SG upon reconstitution into liposomes (Pikula, Hayden, Awasthi, Awasthi & Zimniak, 1994a, b). Kondo *et al.* (1993) reported isolation of DNP-SG-ATPase of (subunit?) molecular weight of 28 000 from human erythrocyte membranes. Apparently, the question of identity of the transporter of DNP-SG conjugates is still open.

Active transport of GSSG and glutathione S-conjugates has been demonstrated in plant (barley mesophyll) cells. However, as opposed to animal cells, in the plants the transport is not outside the cell but into the vacuoles (Martinoia, Grill, Tommasini, Kreuz & Amrhein, 1993; Tommasini, Martinoia, Grill, Dietz & Amrheim, 1993).

Apart from the identification, isolation and molecular characterization of the transporter(s) for GSSG and glutathione S-conjugates, several questions concerning the transport of these compounds should be answered, among them the questions of:

(i) The relation between the transporter(s) of GSSG and of glutathione S-conjugates. Transport characteristics of GSSG and glutathione S-conjugates are similar. Activation energy for transport is 50–60 kJ/mol in both cases (Bartosz *et al.*, 1993; Eckert & Eyer, 1986). One could expect that GSSG inhibits competitively the transport of glutathione S-conjugates and vice versa. Data concerning the erythrocyte membrane are controversial. Kondo, Mura and Taniguchi (1982) found competitive inhibition of low-affinity GSSG transport by DNP-SG and no effect of this compound on the high-affinity GSSG transport. LaBelle, Singh, Srivastava & Awasthi (1986b) did not observe any inhibition of (low affinity) DNP-SG efflux by GSSG. We (Akerboom *et al.*, 1992) and others (Heijn *et al.*, 1992) found a competitive inhibition of high-affinity DNP-SG transport by GSSG and vice versa, and no interaction between the low-affinity transport od GSSG and DNP-DG. One can therefore postulate the presence in the erythrocyte membrane of one high-affinity active transporter of GSSG and DNP-SG, and of two separate low-affinity transporters for GSSG and DNP-SG (Fig. 2).

(ii) The question of tissue specificity of the transporters. Differences in the values of kinetic parameters of the transport in various tissues (Table 2) may indicate a lack of identity of the transporters in these tissues. Even more convincing might be the lack of impairment of GSSG and

Table 2. Characteristics of GSSG- or DNP-SG-stimulated ATPases from human erythrocyte membranes.

Parameter	High-affinity GSSG-ATPase	Low-affinity GSSG-ATPase	DNP-SG- ATPase	Source
K_m (GSSG)	150 μM	2,0 mM	no activity	Kondo *et al.*, 1987
K_m (DNP-SG)	?	?	49 μM	Sharma *et al.*, 1990
	110 μM			Awasthi *et al.*, 1991
K_m (ATP)	80 μM	140 μM		Kondo *et al.*, 1987
p-Chloromercuribenzoate, 100 μM				
% activity	113	66		Kondo *et al.*, 1987
n-Ethylmaleimide, 1 mM,				
% activity	97	57		Kondo *et al.*, 1987
Iodacetamide, 1 mM,				
% activity	88	60		Kondo *et al.*, 1987
Apparent molecular weight				
	82 000	62 000		Kondo *et al.*, 1989
			38 000	Sharma *et al.*, 1990
			28 000	Kondo *et al.*, 1993

Table 3. Characteristics of the two components of DNP-SG transport across the erythrocyte membrane.

Parameter	High-affinity component	Low-affinity component	Source
K_m (DNP-SG)		0.29 mM	LaBelle *et al.*, 1986
		0.94 mM	Kondo *et al.*, 1982
	2.7 μM	0.90 mM	Bartosz *et al.*, 1993
	1.4 μM	0.70 mM	Eckert & Eyer, 1986
K_m(ATP)		1.0 mM	LaBelle *et al.*, 1986
		0.76 mM	Kondo *et al.*, 1982
	32 μM	83 μM	Akerboom *et al.*, 1992
V_m		13 nmol/(mg protein×h)	LaBelle *et al.*, 1986
		183 nmol/(ml vesicles×h)	Kondo *et al.*, 1982
	6.7 nmol (mg protein×h)	94 nmol/(mg protein×h)	Bartosz *et al.*, 1993
	54 nmol/(ml cells×h)	270 nmol/(ml cells×h)	Eckert & Eyer, 1986
pH optimum	7.0	8.0	Bartosz *et al.*, 1993
N-Ethylmaleimide, 1 mM, % activity			
	95	25	Akerboom *et al.*, 1992

DNP-SG export in erythrocytes of patients with the Dubin-Johnson syndrome and of TR⁻ rats (Board, Nishida, Gatmaitan, Che & Arias, 1992). The Dubin-Johnson syndrome in humans and the TR⁻ mutation in rats are characterized by defective transport of DNP-SG and GSSG in the canalicular regions of the hepatocyte plasma membranes (Kitamura, Jansen, Hardenbrook, Kamimoto, Gatmaitan & Arias, 1990; Nishida, Hardenbrook, Gatmaitan & Arias, 1992). However, other authors reported a two-fold decrease in the maximal velocity of GSSG transport in erythrocytes of TR⁻ rats (Heijn *et al.*, 1992).

(iii) The relationship between the high-affinity and low-affinity transport systems in erythrocytes. The high-affinity and the low-affinity transport systems for GSSG (Kondo *et al.*, 1980; 1981) and, apparently, for glutathione S-conjugates, correspond to different proteins. While the high-affinity systems can deal effectively with the low concentrations of GSSG and glutathione S-conjugates, respectively, which can be formed in cells, the function of the low-affinity systems is less clear. Perhaps they constitute an emergency system operating under conditions of severe oxidative or chemical stress.

(iv) Specificity of the transport system(s) for glutathione S-conjugates. Apart from conjugates of hydrophobic organic molecules, also glutathione/cisplatin complex is transported by the glutathione S-conjugate pump; this transport is inhibited by DNP-SG (Ishikawa & Ali-Osman, 1993).

The transport of DNP-SG in erythrocytes is inhibited competitively not only by other glutathione S-conjugates but also by structurally not related hydrophobic anions like organic sulphates, glucuronides and bilirubin ditaurate, the low-affinity transport system being inhibited by a broader class of compounds (Bartosz *et al.*, 1993). The DNP-SG ATPase is also stimulated by bilirubin ditaurate, estradiol glucuronides and bile acid sulphates and glucuronides (Singhal, Sharma, Gupta, Ahmad, Zimniak, Radominka, Lester & Awasthi, 1991; Zimniak & Awasthi, 1993). On the other hand, canalicular vesicles of hepatocytes actively transport glucuronides and this transport is inhibited by DNP-SG (Kobayashi, Komatsu, Nishi, Hara & Hayashi, 1991). Perhaps the transport system for glutathione S-conjugates (the "glutathione S-conjugate export pump") (Ishikawa, 1992) is a "hydrophobic anion export pump" of low specificity? The specificity of this system may even be not confined to anions since a recent report claims that in the erythrocyte (lacking the multidrug transporter) this system can also transport such substrates of the multidrug transporter as doxorubicin, daunomycin and vinblastine, and that isolated DNP-SG-ATPase is stimulated also by doxorubicin (Awasthi, Singhal, Gupta, Ahmad, Zimniak, Radominska, Lester & Shrama, 1994).

(v) The mechanism of action of the "glutathione S-conjugate export pump". It is not clear how such a low specificity transport system can recognize and recruit its substrates; in this respect it might be similar to the multidrug transporter but the precise mechanism of action is yet only a matter of speculation in both cases.

(vi) Physiologic role of the "glutathione S-conjugate export pump".

Apart from the obvious role in detoxication, the pump may also function in the physiologic export of metabolites. One such conjugate is leukotriene C_4, formed a result of glutathione conjugation with leukotriene A_4, catalysed by microsomal leukotriene C_4 synthetase. Leukotriene C_4 is a precursor of leukotrienes D and E formed by the action of γ-glutamyltransferase and dipeptidase, respectively, in the extracellular space. Export of leukotriene C_4 by the "glutathione S-conjugate export pump" is therefore an important factor in the mechanism of action of cysteine leukotrienes.

Other compounds suspected to be transported by this system include some hepoxilins and prostaglandins (Ishikawa, 1992).

Apparently, the role of the transport system called tentatively the "glutathione S-conjugate export pump" is broader and more important, even in the absence of exogenous challenge, than thought originally.

Acknowledgement

This study was supported from Grant No. 6 P203 001 04 of the Committee for Scientific Research (Poland).

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Current Topics in Biophysics, vol. 18(2), 238-244

ON THE ROLE OF HYDROXYALKENALS IN INFLAMMATION

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(Accepted 28 December 1994)

4-Hydroxynonenal (HNE) and other hydroxyalkenals are widely considered as cytotoxic and genotoxic derivatives of polyunsaturated fatty acids produced by free radical reactions. But this class of lipid peroxidation products has several other remarkable biological properties, e.g. they belong to a group of lipid mediators of inflammation. It was shown that HNE acts as a chemoattractant towards rat neurophils and as a chemokinetic agent towards human neutrophils, both *in vitro*. Using *in vivo* model — the Sephadex model of acute traumatic inflammation — it was proven that HNE is chemotactic also *in vivo*. HNE is produced by neutrophils themselves during phagocythosis and its concentration in the inflammatory site correlates with the intensity of the inflammatory response in terms of ingress rate of neutrophils and intensity of the oxygen burst. Linner and coworkers produced specific antibodies which recognize protein adducts of HNE. By means of these antibodies they were albe to localize HNE adducts in human neutrophils by elektron microscopy. We suggest that HNE is part of an autocatalytic cycle whereby neutrophils which immigrate into an inflammatory produce HNE which stimulates the ingress of new neutrophils. A possible molecular basis for the chemotactic activity of HNE whill be discussed.

Dedicated to Professor Hermann Esterbauer on the occassion of his 60th birthday.

SCOPE

It is the aim of this contribution to present experimental evidence from our laboratories and others which support the idea of an autocatalytic cycle operating in acute inflammation, which involves reactive oxygen species (ROS), lipid-derived reactive aldehydes and neutrophilic polymorphonuclear granulocytes. This cycle results in an augmentation of the inflammatory process.

HNE AS SECOND MESSENGER OF ROS ACTIVITY

Neutrophils exhibit a so called oxidative burst, i.e. they produce superoxide when stimulated by a variety of soluble and particulate stimuli, including phorbol myristate acetate (PMA) and heat-killed opsonized bacteria like *Staphylococcus aureus*. Superoxide anion and other ROS generated by activated neutrophils can react with a number of tissue targets, including polyunsaturated fatty acids (PUFAs) of neighboring cell membranes. It has been established by Shohet *et al.* (Shohet, Pitt & Bahner, 1974) and Stossel *et al.* (Stossel, Mason, & Smith, 1974), that lipid peroxidation does occur in neutrophils. Lipid peroxidation – resulting from free radical chain reactions initiated by ROS-attack – produces initially peroxidized free fatty acids and phospholipids which subsequently break down into short chain cleavage products such as alkenals and hydroxyalkenals. One such product is 4-hydroxynonenal (HNE) which is derived from omega-6-polyunsaturated fatty acids, such as linoleic, linolenic and arachidonic acid. It shows several remarkable biological properties (for reviews see Zollner, Schaur & Esterbauer, 1991; Schaur, Zollner & Esterbauer,1991). We have shown recently that HNE is produced in the micromolar range from liposomes by a pathway which depends on myeloperoxidase isolated from neutrophils (Stelmaszynska, Kukovetz, Egger & Schaur, 1992).

The data reported here are primarily related to the role of HNE and other hydroxyalkenals as chemoattractants for neutrophils (An earlier review by Curzio appeared in 1988).

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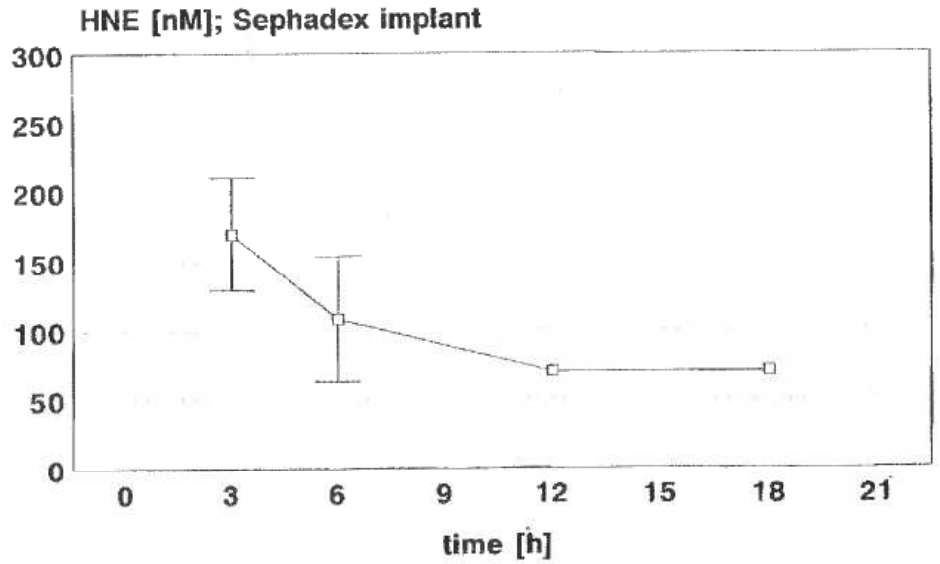

Fig. 1. Formation of HNE by stimulated neutrophils *in vitro*: dependence on inflammation time (from Schaur *et al.*, 1994). At zero time an inflammation was provoked in rats by injection of Sephadex gel and at the time intervals indicated the neutrophils were collected, stimulated with Zymosan to phagocytosis and incubated at 37°C. 35 min after stimulation HNE was determined. For the reason of comparison the concentrations given refer to the Sephadex implant; time intervals (hrs) and number of animals (in brackets): 3 (3); 6 (3); 12 (2); 18 (1).

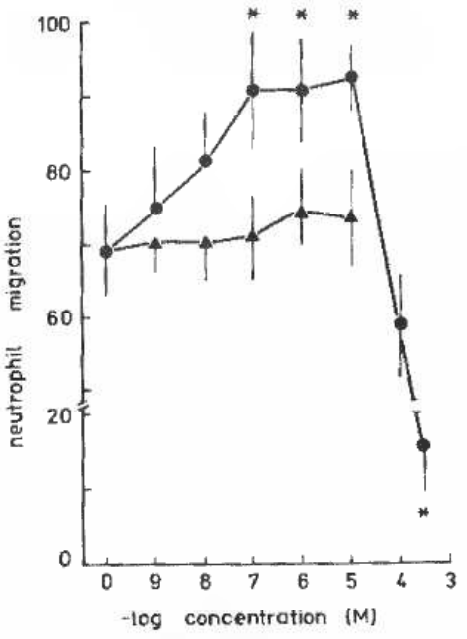

Fig. 2. Effect of increasing concentrations of HNE (circles) and nonanal (triangles) on the oriented migration of rat neutrophils (from Rossi *et al.*, 1991). The neutrophils, placed in the upper compartment of a perspex modified Boyden chamber, were incubated for 75 min at 37°C. The results are expressed in um. A representative experiment performed in quadruplicate is shown. Values significantly different from control values: * $p < 0.001$.

IN VITRO STUDIES

Occurence of HNE in neutrophils and formation during phagocytosis

When rat neutrophils were isolated from an inflammatory focus and stimulated with Zymosan, they were able to produce HNE in vitro depending on the time of isolation (Fig. 1). The highest production of HNE (0.17 µM) by phagocyting neutrophils was observed at the shortest inflammation time studied (3 hrs).

In order to localize HNE bound to protein within human neutrophils Linner *et al.* (Linner, Buescher, Siemsen, Dratz, Quinn & Jesaitis, 1992) performed immunocytochemical studies by electron microscopy. They produced specific antibodies, that recognize HNE adducts of bovine serum albumin (BSA), but not native underivatized BSA. In resting cells the anti-HNE antibodies were localized to the membrane and cytoplasm with preponderance in the surface and organelle membranes. During active phagocytosis of opsonized *S. aureus*, neutrophils demonstrate a translocation of the membrane components of the superoxide generating system to the membrane of the phagosome. Clearly visible were grains denoting the presence of HNE adducts juxtaposed to the bacterial cell surface.

Chemotaxis in vitro

HNE and other hydroxyalkenals have been shown to stimulate oriented migration of neutro-

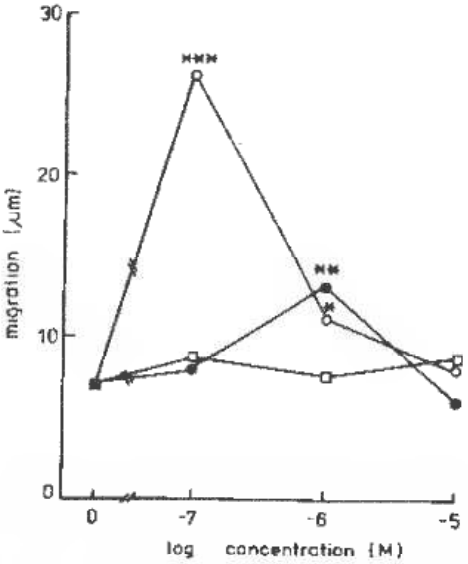

Fig. 3. Chemokinetic activity of HNE (filled circles), 2-nonenal (empty circles) and nonanal (empty squares) (from Curzio *et al.*, 1994). Neutrophils were preincubated with 1.6 mM N-ethyl-maleimide at 4°C for 15 min, washed twice and resuspended in Hanks' solution + 2% BSA at 4 x 10^6 cells per ml. Cell random migration was assayed by Boyden chambers. The asterix indicate the significance with respect to the control (filled squares): ***$p < 0.002$; ** $p < 0.02$, * $p < 0.05$.

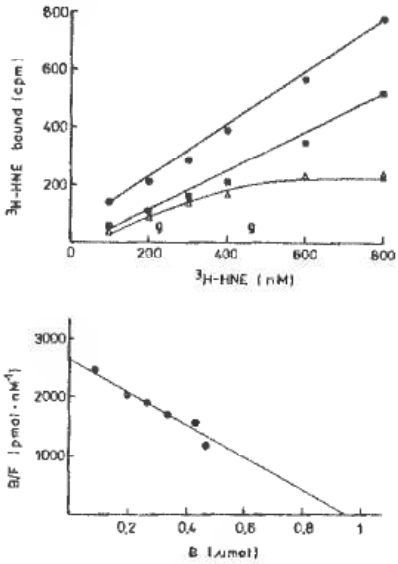

Fig. 4. Saturation curve of (^{3}H)-HNE binding to human neutrophils (from Curzio *et al.*, 1994) (Upper): Neutrophils (0.6 x 10^6) were preincubated with 1.6 mM N-ethyl-maleimide at 4°C for 15 min, washed twice and incubated at 4°C for 30 min with increasing concentrations of (^{3}H)-HNE in the presence (squares) or absence (circles) of 3.2 mM unlabelled HNE. Specific binding (triangles) was determined by the difference between the two curves. (Lower) Scatchard plot of (^{3}H)-HNE specific binding. Shown is a representative experiment performed in duplicate.

phils of rats (Curzio, Esterbauer & Di Mauro, 1986b; Rossi, Curzio, Di Mauro, Fidale, Garramone, Esterbauer, Torrielli & Dianzani, 1991). HNE in concentrations between 0.1 and 10 micromolar induced a significant stimulation of cell oriented migration while the saturated aldehyde nonanal was ineffective. Maximal stimulation by HNE (about 40 % above control values) was obtained by 1 micromolar HNE (Fig. 2). Concentrations above 0.1 millimolar had an inhibitory effect and 1 millimolar HNE completely inhibited cell motility.

The effect was only observed in the presence of BSA. Since the aldehydes easily react with the thiol group of BSA, the chemotactic agent could either be the free aldehyde or the BSA-HNE adduct.

Chemokinesis in vitro

In contrast to rat neutrophils HNE in micromolar concentration stimulates random migration of human neutrophils (Fig. 3) (Curzio, Esterbauer, Di Mauro & Dianzani, 1990; Curzio, Ferretti, Stephens, Esterbauer & Dianzani, 1994). Investigations of this chemokinetic mechanism indicate that (^{3}H)-HNE binding to neutrophils results both in non-specific bonds to the thiol groups of neutrophils and in binding to a saturable, reversible and specific unsaturated-aldehyde-site (Curzio *et al.*, 1994). Scatchard analysis revealed that there is a single binding site with an apparent affinity constant of 319 nM and a density of 1.57 pmoles per million cells (Fig. 4).

The relative potencies of HNE, 2-nonenal, and nonanal as chemokinetic agents were compared with their relative abilities to compete with (^{3}H)-HNE for specific binding. The order of potencies in competing for (^{3}H)-HNE binding parallels their order of potency as chemokinetic agents: 2-nonenal > HNE >> nonanal. Therefore the hydroxy-group does not seem to be decisive for activity. The kind of interaction between unsaturated aldehydes and specific binding sites is as yet unknown.

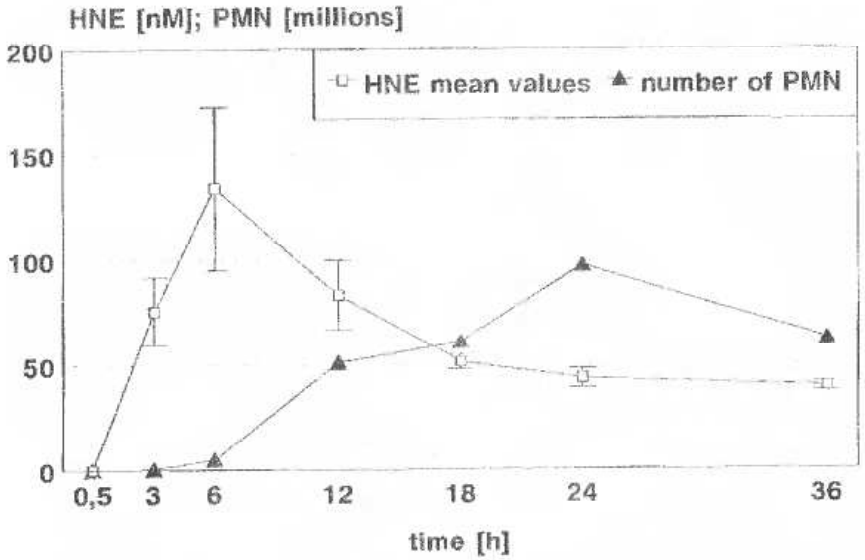

Fig. 5. Time course of neutrophils and HNE in the inflammatory focus of Sephadex-treated rats (from Schaur *et al.*, 1994). At zero time an inflammation was provoked in rats by injection of Sephadex gel and the concentration of HNE and the number of neutrophils in the inflammatory focus were determined, time intervals (hrs) and the number of animals (in brackets): 0.5 (1); 3 (3); 6 (4); 12 (3); 18 (3); 24 (3); 36 (3).

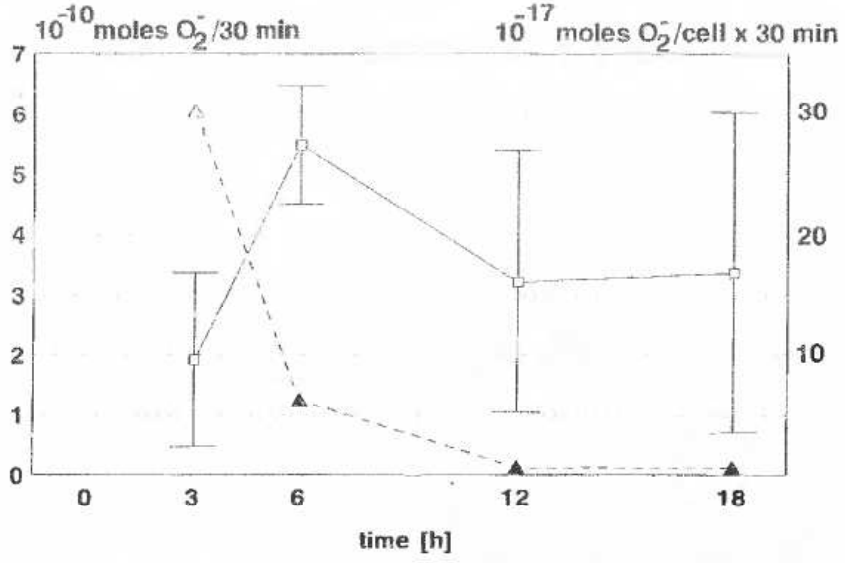

Fig. 6. Production rate of superoxide by neutrophils *in vitro*: dependence on inflammation time (from Schaur *et al.*, 1994). At zero time an inflammation was provoked in rats by injection of Sephadex gel and at the time intervals indicated the neutrophils were collected; the production rate of superoxide anion was determined for an incubation period of 30 min at 37°C; full line: total production of superoxide anion within 30 min; broken line: production within 30 min per single neutrophil.

IN VIVO STUDIES

Occurrence in vivo

HNE was first detected in pleural exudates of rats (Curzio, Poli, Esterbauer, Biasi, Di Mauro & Dianzani, 1986a) indicating its occurrence during inflammation *in vivo*. Subsequently the question was studied, whether HNE is formed during the ingress of neutrophils in the Sephadex model of inflammation (Schaur *et al.*, 1994).

The polydextrane Sephadex G-200, which causes an acute aseptic traumatic inflammation, was injected subcutaneously into rats. The implants were excised 6 – 36 hours later, and the neutrophils separated from the exudate by centrifugation. After extraction with dichloromethane

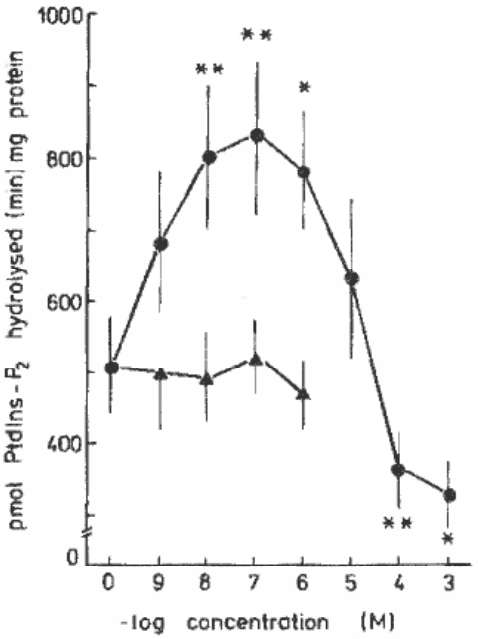

Fig. 7. Effect of increasing concentrations of 4-hydroxynonenal (circles) and nonanal (triangles) on basal PLC activity (from Rossi *et al.*, 1991). The enzymatic activity was determined *in vitro* on plasma membranes isolated from rat neutrophils. Incubations were for 2 min at 37°C. The results are the means ± S.D. of three different experiments performed in duplicate and are expressed as pmoles Ptd-P_2 hydrolyzed per min and mg protein. In comparison with the basal activity: $^{**}p < 0.01$; $^{*}p < 0.05$.

HNE was identified in the exudate by non-derivative reversed phase HPLC in combination with on-line uv-spectroscopy. Its concentration was found to be sufficiently high to show a chemotactic effect *in vitro* The concentration of HNE in the inflammatory focus did not correlate - as one would expect - with the number of neutrophils present (Fig. 5). While the peak of HNE coincided with the time point of the highest turnover rate of neutrophils (0.13 µM at 6 hrs after implantation), the highest number of neutrophils (about 100 million cells) occurred not earlier than 18 hrs later (24 hrs after onset of inflammation).

An explanation for the relatively early maximum of the HNE concentration at the site of inflammation (6 hrs) compared with the peak of neutrophils (24 hrs) can be obtained by taking into account the time dependance of the turnover rate of neutrophils. At about 6 hrs after onset of inflammation both the rates for ingress (52 million cells per hour) and decay of neutrophils show a peak resulting in a minimum of half life (about 20 min) (Egger, 1988). This suggests that HNE is produced by the process of autoxidative selfdestruction of neutrophils.

Chemotaxis in vivo

When applied exogenously HNE was able to augment the number of neutrophils in the inflammatory focus. The concentration necessary for this *in vivo* effect was considerable higher than the effective concentration for chemotaxis *in vitro*. Synthetic HNE was added in a concentration of 1 and 10 µM respectively to the Sephadex gel prior to injection. While 1 µM HNE did not change the number of neutrophils found after an inflammation time of 6 hrs in the gel, the number of neutrophils was 3-fold higher than in the untreated control animals, when 10 µM HNE was applied $(23.2 \pm 10.5) \cdot 10^6$ cells versus $(7.5 \pm 1.8) \cdot 10^6$ cells; $n = 5$).

The concentration for half maximal stimulation of oriented migration *in vitro* was found to be 0.14 µM (Curzio *et al.*, 1986b). The difference may be due to the fact that most likely the greater part of HNE is retained within the Sephadex particles, while the neutrophils accumulate in the outer shell of the gel. Moreover at the boundary of the gel to the surrounding tissue HNE will diffuse away so

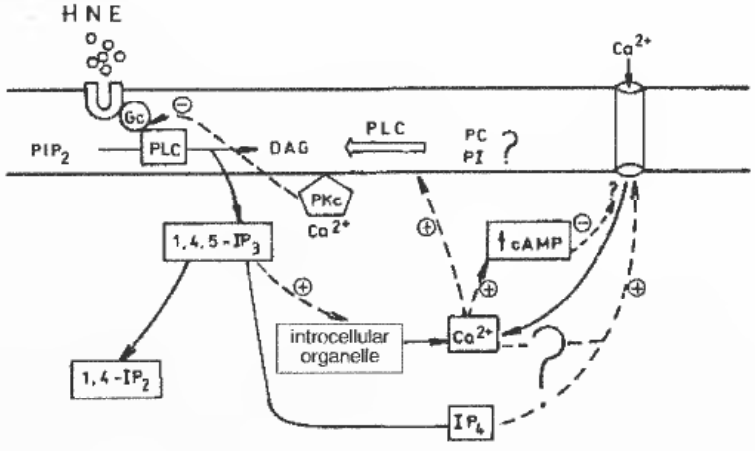

Fig. 8. Model for the regulation of leukocyte responses to chemoattractants (adopted from Uhing *et al.*, 1988). Metabolic pathways are represented by (——) and regulatory steps by (- - - -), respectively. Areas that have not been definitely established include a question mark (?).

that more HNE is need to build up a gradient comparable to stable *in vitro* conditions.

The production of HNE seems to be tightly connected to the oxidative burst of neutrophils. It was found that the formation of HNE correlated strongly with the production rate of superoxide anion at the site of inflammation (Schaur, Dussing, Kink, Schauenstein, Posch, Kukovetz & Egger, 1994).

The formation of superoxide was also measured by the cytochrome c reduction assay *in vitro* (Fig. 6). The maximum of the production rate of superoxide anion was observed at the same inflammation time (6 hrs), when the HNE maximum occurred. Cells which ingressed earliest (at 3 hrs) showed the highest production rate of superoxide per cell ($307 \cdot 10^{-18}$ moles per cell and 30 min).

Molecular model of action

Taken together our results support the idea that HNE is part of an autocatalytic cycle of inflammation whereby phagocytic neutrophils immigrate into an inflammatory focus and produce HNE as a consequence of the oxidative burst and HNE in turn may stimulate the ingress of further neutrophils.

Evidence indicates a central role of phosphoinositide metabolism, calcium mobilization, and protein kinase C activation in mediating HNE-stimulated function.

It has been shown by Rossi *et al.* (1991) that HNE activates phospholipase C (PL-C) of neutrophils to produce 1,4.5-IP_3 and diacylglycerol (DAG), whereas nonanal is ineffective (Fig. 7).

A synergism between HNE and GTP suggests that acceleration of phosphatidyl-inositol-4,5-biphosphate breakdown induced by HNE is mediated by a regulatory G protein, most likely the pertussis/cholera-toxin-sensitive GTP-binding protein termed G_e (Fig. 8). In addition to calcium release from intracellular stores, sustained PIP_2 hydrolysis leads to enhanced membrane calcium permeability and a secondary formation of diacylglycerol from a precursor other than the phosphoinositides. These latter phenomena are well correlated with activation of the respiratory burst (Uhing, Dillon, Polakis, Truett & Snyderman, 1988).

Chemoattractant-induced cellular activation can be attenuated by several mechanisms (Uhing *et al.*, 1988):

Agonists can be hydrolyzed externally or internally and degraded within leukocytes.

The receptor can also be down regulated by internalization and an additional agonist-specific mechanism.

Translocation and activation of protein kinase C disrupts the coupling of G_e and PL-C.

Elevated cAMP levels, induced by either the calcium-dependent mechanism involved in chemoattractant-elicited responses or by hormones that act through adenylate cyclase, also attenuate the chemoattractant-induced activation of neutrophils.

For HNE degradation is a very probable mechanism in addition to binding to thiol groups of proteins. The metabolic pathways of HNE in neutrophils have not yet been studied, but it is known from other cell types that it can be oxidized to its carboxylic acid, reduced to its di-hydroxy-derivative and conjugated to glutathione (Esterbauer, Zollner & Schaur, 1990).

It should be stressed, that very likely there are additional molecular mechanisms for the role of HNE in the inflammatory process. Since HNE is known to stimulate adelylate cyclase in other tissues (Paradisi *et al.*, 1985) this may also be the case in neutrophils.

Moreover, other events in neutrophil activation may be modulated by HNE. It has been shown that HNE promotes the adhesion of leukocytes to endothelial cells (Zimmerman *et al.*, 1993).

And last, but certainly not least, other inflammatory cells like endothelial cells may also play a role. Recently Natarajan *et al.* (1993) reported on the five-fold stimulation of phospholipase D (PL-D) activity in vascular endothelial cells by HNE. PL-D is involved in an alternative pathway for the generation of DAG. It catalyzes the hydrolysis of membrane phospholipids to phosphatidic acid (PA) with the subsequent hydrolysis of PA by a phosphatase to produce DAG. The exact mechanism by which HNE and other hydroxyalkenals activate PL-D is not clear and the potential physiological relevance of PA/DAG generation as a consequence of HNE-induced PL-D activation is as yet unknown. The author speculate that the PL-D mediated generation of PA and subsequent conversion to DAG may represent an important signal transduction pathway in endothelial cells in response to agonists and other bioactive substances including oxidants and membrane lipid peroxidized byproducts.

So it may turn out that we are just at the onset of clarifying the complex role hydroxyalkenals in inflammation.

Acknowledgements

The support by the Association for International Cancer Research, St. Andrews, Scotland, and by the Italian Ministry for Universities, Scientific and Technological Research, is gratefully acknowledged.

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