Acta Biochimica Polonica

Vol. 47 No. 4/2000

963–971

QUARTERLY

In vitro effects of ozone on human erythrocyte membranes: An EPR study

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Received: 24 July, 2000; revised: 24 October, 2000; accepted: 15 November, 2000

Key words: medical ozone, erythrocyte membrane fluidity, electron paramagnetic resonance, spin labelling

The effects of ozone at different concentrations (10, 30, 45 g/m^3) on fluidity and thermotropic properties of erythrocyte membranes were investigated by EPR using two spin probes: 5-doxylstearic acid (5-DSA) and 16-doxylstearic acid (16-DSA). The effect of ozone on the erythrocyte membrane fluidity was a dose-dependent process. The ozone at concentration of 10 g/m^3 caused rigidization of the membrane while at concentration of 45 g/m^3 increased fluidity both on the surface and in the deeper hydrocarbon region of the membrane. Temperature transitions close to the polar heads region (monitored by 5-DSA) were not sensitive to an increase in ozone concentration. In the case of 16-DSA, low temperature thermotropic transition (around 20°C) gradually decreased with the increase of ozone concentration. High temperature transition (around 40°C) significantly differed at the ozone concentration of 10 g/m^3 and 45 g/m^3, being higher and lower, respectively, as compared to untreated cells. For the ozone concentration of 45 g/m^3 the disappearance of the low temperature break and the appearance of two breaks at 37°C and 16°C were observed.

Autotransfusions of ozonized blood or infusions of gaseous ozone into blood vessels and body cavities are believed to exert therapeutic effects in some pathological states (Shiratori *et al.*, 1993; Washuttl, 1988; Burgard, 1990). In medical applications an oxygen/ozone mixture (the so called medical ozone) is used at concentrations ranging from 1 to 40 μg ozone per 1 ml of oxygen.

Ozonotherapy is criticised by numerous investigators, on the grounds that the mechanism of therapeutic ozone action is still unknown (Mehlman & Borek, 1987; Oepen, 1992). Therefore, investigations on the reac-

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Abbreviations: 5-DSA, 5-doxylstearic acid; 16-DSA, 16-doxylstearic acid; EPR, electron paramagnetic resonance; NaCl/P_i, phosphate-buffered saline.

tion of ozone with biological molecules and membrane structures are a subject of crucial importance.

The available toxicological information concerning ozone consists of studies on ozone's effect on model cells such as human or animal erythrocytes. Exposure of erythrocytes to ozone can result, among others, in increased osmotic fragility of the cell membrane and loss of membrane enzyme activities (Chan *et al.*, 1977). Special attention of investigators was focused on the reaction of ozone with lipids and proteins as they are components of the plasma membrane (Mudd *et al.*, 1997; Freeman *et al.*, 1979; Ignatenko & Cherenkevich, 1985; Verweij *et al.*, 1982).

Ozone is known to produce free radicals both *in vitro* and *in vivo* (Mudd, 1977; Menzel, 1970). The oxidation of phospholipids by ozone has been studied thoroughly (Mudd *et al.*, 1997; Freeman *et al.*, 1979). The reaction takes place at the fatty acid double bonds in a manner consistent with the mechanism of Criegee (1975). Wróbel *et al.* (1999) using nitroxide spin probe TEMPO embedded in ozonized erythrocyte membrane have shown that a decrease of EPR signal was due to recombination of nitroxide radicals with organic free radicals produced both in the process of lipid peroxidation and ozonolysis of double bonds. The oxidation of amino acids and proteins has also been extensively studied (Mudd *et al.*, 1969; Ignatenko & Cherenkevich, 1985; Verweij *et al.*, 1982; Matus *et al.*, 1987). Uppu *et al.* (1995) reported that both lipids and proteins of erythrocyte membranes were oxidized after exposure to ozone. Wróbel and co-workers (1997) have shown that structural changes in erythrocyte membranes treated with ozone may be related mainly to a specific modification caused by ozone in cytoskeletal proteins.

The reaction of ozone with biological membranes has been the subject of enduring controversy. It has been reported that lipid peroxidation caused by ozone decreases as well as increases the molecular ordering of biological membranes (Bruch & Thayer, 1983; Bartosz *et al.*, 1987; Van Ginkel & Sevanian, 1994; Richter, 1987). On the other hand, Kaler *et al.* (1989), using fluorescent probes inserted in ozonized ghosts, have shown that ozone decreases the microviscosity of boundary lipid and increases microviscosity of bulk lipid in the bilayer.

We have not found, in available literature, investigations on the influence of ozone on the order–disorder transitions in cell membranes. Therefore, the aim of our investigations was to assess the effects of ozone on fluidity and thermotropic phase transitions of human erythrocyte membrane.

The measurement of temperature-induced phase transitions is one of the most powerful tools for analysing dynamics of the processes occurring in the cell membrane. Structural changes involved in these transitions are still unclear due to the high level of complexity reflected in a high level of anisotropy of these membranes. However, noteworthy is the lateral mobility of glycoproteins which changes discontinuously with temperature (Nigg & Cherry, 1979) and appears to be controlled by cytoskeletal proteins (Golan & Weatch, 1980; Forte *et al.*, 1985).

Membrane fluidity has been studied by the electron paramagnetic resonance (EPR) method. In order to investigate the effect of ozone at different concentrations on erythrocyte membranes two doxyl stearate spin probes, 5-DSA and 16-DSA, which differ in the location of nitroxide free radical inside the bilayer, were used. This allowed to obtain information about the fluidity of different regions of the membrane. The spin probe 5-DSA monitors membrane lipid mobility close to the headgroup area whereas 16-DSA gives information about the hydrophobic core.

MATERIALS AND METHODS

Fatty acid spin labels: 5 doxylstearic acid (5-DSA) and 16 doxylstearic acid (16-DSA)

were purchased from Sigma (St. Louis, MO, U.S.A.). For each spin label stock solutions (0.1 mg/ml in ethanol) were prepared and kept at –80°C.

Blood was obtained from healthy volunteers by vein puncture and mixed with 1/10 volume of 0.13 M trisodium citrate. Erythrocytes were isolated from fresh blood by centrifugation at 4°C, at 1500 × ***g*** and purified by three cycles of resuspension in and washing with NaCl/P_i (phosphate-buffered NaCl solution, 310 mOsm, pH 7.4), after careful removal of the buffy coat.

Ozone was generated by passing pure gaseous oxygen at 30 l/h through an apparatus (ATO3, Krio Metrum, Poland) producing silent electric discharges. Ozone concentration was controlled by the iodometric method (Mudd *et al.,* 1969; Koonotz & Heath, 1979) assuming that 1 μmol O_3 was corresponded to 1 μmol J_2. The ozone/oxygen mixture (range of concentrations: 10–45 g/m^3 of O_2/O_3 mixture) was passed for 3 min over 5 ml of stirred blood sample in NaCl/P_i.

Labelling was performed on a thin film of the spin probe formed by evaporation of 150 μl of stock solution under a flow of nitrogen gas in Eppendorf tube (Ogura *et al.*, 1988).

Erythrocytes, 50 μl, were put into a test tube containing a thin film of the label and incubated at 37°C for 15 min. After being washed twice with 5 ml of NaCl/P_i, the labelled erythrocytes were put into a quartz capillary tube and placed in the cavity of EPR Radiopan spectrometer.

EPR spectra were obtained at X-band (9.4 GHz), at modulation frequency of 100 kHz. The scan time was 4 min, and the time constant 0.3 s. The temperature of the samples was changed in the range of 5–50°C with the accuracy of ± 0.5°C at the site of the sample.

Generally, the fluidity of the membrane can be estimated from the order parameter S which can vary between 0 and 1. Changes of the order parameter values correspond with changes of membrane local viscosity – its increase means a decrease of viscosity.

The S parameter was calculated from the formula of Gaffney (1976):

$$S = \frac{A_{II} - (A_{\perp} + C)}{A_{II} + 2(A_{\perp} + C)} \times 1.723 \qquad (1)$$

$C = 1.4 - 0.053\,(A_{II} - A_{\perp})$

where $2A_{II}$ and $2A_{\perp}$ are parallel and perpendicular hyperfine splitting parameters of the spectrum, respectively (Fig. 1).

Due to the anisotropy of the erythrocyte membrane, the calculated order parameters are not true order parameters (Schreier *et al.*, 1978). Therefore, in our experiments, the parallel hyperfine splitting parameter was used to obtain information on the dynamic behaviour of the membrane.

The rotational correlation time τ_c for a rod-like molecule may be calculated according to the formula of Hemminga (1975):

$$\tau_c = K \cdot \Delta W_0[(h_0/h_{-1})^{1/2} - 1] \qquad (2)$$

$K = 6.5 \cdot 10^{-10}\ s \cdot G^{-1}$

where ΔW_0 is the peak-to-peak linewidth of the central line, h_0, and h_{-1} are the peak height of the central and high-field lines, respectively (Fig. 1).

The changes in the freedom of motion of 16-DSA with temperature were analysed by the parameter $\log(h_0/h_{-1})$ described by Minetti *et al.* (1984). At each temperature five spectra were recorded, and standard deviations of the measurements of the peak height of the central (h_0) and high-field lines (h_{-1}) were ± (0.03 – 0.10).

RESULTS

The EPR spectra obtained at 37°C for 5- and 16-DSA in erythrocyte membranes are presented in Fig. 1. All spectra were recorded 10 min after labelling of the sample. From the spectra of untreated and ozonized erythrocytes the values of order parameter (S), corre-

lation time (τ_c) and $\log(h_0/h_{-1})$ parameter were calculated (Table 1). The values of S and

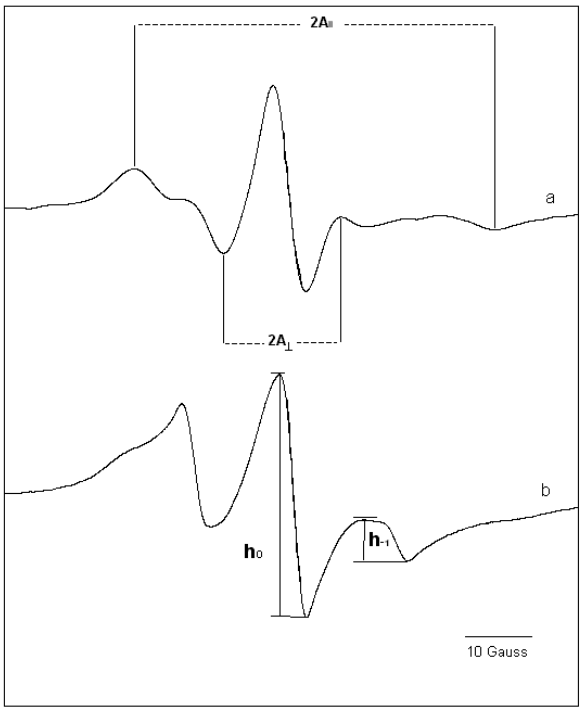

Figure 1. Typical EPR spectra of 5-doxylstearic acid (a) and 16-doxylstearic acid (b) labelled erythrocytes.

On the upper plot the maximum hyperfine splitting parameter is shown. On the lower plot the spectral amplitudes of the central and high-field peak are indicated by h_0 and h_{-1}, respectively.

τ_c for 5-DSA and 16-DSA were slightly increased at the ozone concentration of 10 g/m^3. This change was, however, statistically insignificant. Ozone at higher concentrations (30 and 45 g/m^3) produced detectable decreases in both correlation times and order parameters of the spin probes. Differences in S and τ_c for 5-DSA at ozone concentration of 30 g/m^3 were statistically insignificant. The order parameters of the 45 g/m^3 ozone treated cells were found to decrease by 3.0 ± 0.9% for 5-DSA and 5.3 ± 1.0% for 16-DSA, respectively, compared to that of untreated erythrocytes.

To study thermotropic properties of ozonized erythrocyte membranes changes of order (S) and motional freedom ($\log(h_0/h_{-1})$) of spin probes 5-DSA and 16-DSA in the 5–50°C temperature range at different ozone concentrations (10, 30, 45 g/m^3) were measured. The data for erythrocyte cells treated with ozone at concentration of 45 g/m^3 compared to untreated cells are presented as plots in Figs. 2 and 3. The plots of $2A_{II}$ against temperature for untreated and ozonized erythrocytes labelled with 5-DSA were composed of two approximately straight lines with a temperature break around 36°C. The curves of $\log(h_0/h_{-1})$ *versus* reciprocal temperature for ozonized erythrocytes labelled with 16-DSA revealed three different temperature regions with two transition temperatures, whereas for untreated cells three transitions were observed at 9 ± 1, 20 ± 1 and 40 ± 1°C. Similar temperature transitions were also observed for untreated erythrocytes by other authors (Minetti *et al.*, 1984; Forte *et al.*, 1985). Changes in the

Table 1. Variation of order parameter (S) and correlation time (τ_c) with ozone concentration at 37°C for the 5- and 16-doxylstearic acid (5-DSA and 16-DSA) labelled erythrocytes

Spin probe	Ozone concentration (g/m^3)	Order parameter (S)	Correlation time (τ_c) ($\times 10^{10}$)
5-Doxylstearic acid	0	0.620 (± 0.003)	20.1 (± 0.1)
	10	0.625 (± 0.004)	20.4 (± 0.3)
	30	0.616 (± 0.008)	19.9 (± 0.4)
	45	0.601 (± 0.005)**	19.1 (± 0.3)**
16-Doxylstearic acid	0	0.151 (± 0.003)	3.33 (± 0.08)
	10	0.154 (± 0.004)	3.39 (± 0.10)
	30	0.145 (± 0.004)*	3.18 (± 0.09)*
	45	0.143 (± 0.002)**	3.15 (± 0.03)*

*$P < 0.05$; **$P < 0.001$ *versus* untreated cells.

calculated transition temperatures due to increased ozone concentration are shown in Table 2. Temperature transitions close to the polar heads region (monitored by 5-DSA) were not sensitive to the increase of ozone concentration. In the case of 16-DSA, the low temperature thermotropic transition (around 20°C) gradually decreased with the increase of ozone concentration. High temperature transition (around 40°C) significantly differed for the ozone concentration of 10 g/m^3 and 45 g/m^3 being higher or lower as compared to untreated cells. For the ozone concentration of 45 g/m^3 the disappearance of the low temperature break and the appearance of two breaks at 37°C and 16°C were observed (Fig. 3).

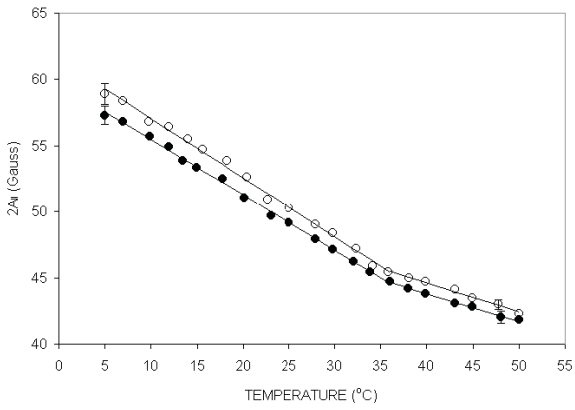

Figure 2. Effect of ozone at concentration of 45 g/m^3 on the thermotropic behaviour of 5-DSA labelled erythrocytes.

(š) Untreated cells; (˜) ozonized cells. Each point represents the mean of 5 repeats; typical results ± S.D. are indicated.

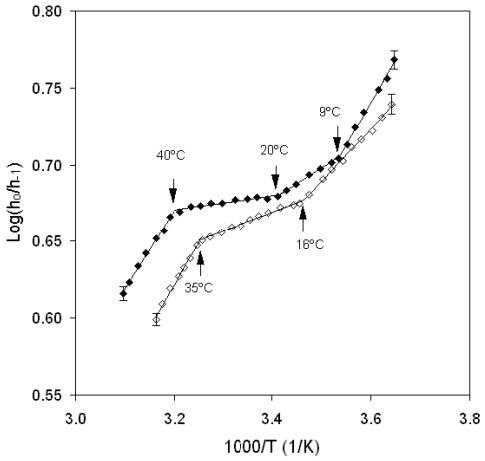

Figure 3. Effects of ozone treatment on the thermotropic behaviour of 16-DSA in erythrocytes.

(¿) Control cells; (¯) cells treated with ozone at concentration of 45 g/m^3. Each point represents the mean of 5 repeats; typical results ± S.D. are indicated.

DISCUSSION

It is well known that ozone is one of the most powerful oxidants. During ozonotherapy plasma membranes may be the major sites of damage (Mustafa, 1990; Mehlman & Borek, 1987; Bolton *et al.*, 1982). The reaction of ozone with membrane lipids takes place mainly with unsaturated fatty acid residues (Pryor, 1994; Pryor *et al.*, 1995). The influence of ozone on membrane lipid fluidity has been a subject of controversy. It has been reported

Table 2. Thermotropic phase transition temperatures for untreated and ozonized erythrocytes

Ozone concentration (g/m^3)	Transition temperature (°C)			
	Low		High	
	5-DSA	16-DSA	5-DSA	16-DSA
0	–	20 (± 1)	36 (± 1.5)	40 (± 1)
10	–	19 (± 2)	35 (± 1)	43 (± 1.5)*
30	–	17 (± 1.5)*	35 (± 2)	39 (± 1)
45	–	16 (± 1)***	36 (± 2)	37 (± 1)**

*P < 0.01; **P < 0.005; ***P < 0.0005 *versus* untreated cells.

that lipid peroxidation causes a decrease as well as an increase in membrane fluidity of biological membranes (Bruch & Thayer, 1983; Bartosz *et al.*, 1987; Van Ginkel & Sevanian, 1994; Richter, 1987).

The results obtained in this work show that the effect of ozone on the erythrocyte membrane fluidity is dose-dependent. A small increase in order parameter (S) and correlation time (τ_c) observed in erythrocyte membrane at ozone concentration of 10 g/m^3 suggests that its lower concentrations probably cause rigidization of the membrane. This result is in agreement with that reported by Wróbel & Gomułkiewicz (1999). Decreased values of τ_c and S of 5-DSA and 16-DSA obtained in erythrocytes for ozone concentration of 45 g/m^3 indicate a decrease in order and increased fluidity both on the surface and in the deeper hydrocarbon region of the membrane. However, the decrease of order parameter and correlation time were more expressed for 16-DSA spin probe. This suggests that the fluidizing effect of ozone at concentration of 45 g/m^3 is less evident on the surface of the bilayer than in the hydrophobic core.

Erythrocyte membranes have been reported, using EPR spin labelling method, to undergo three thermotropic phase transitions at 8, 20 and 40°C. Transitions at 8 and 40°C depend on the interactions of cytoskeletal proteins with membrane; protein 4.1 is involved in the 8°C transition of red blood cells, whereas spectrin seems to be involved in a structural membrane transition at 40°C. The 20°C transition is related to the components of the lipid phase of the membrane (Minetti *et al.*, 1984).

We also observed, using 16-DSA spin label, three temperature transitions at 9 ± 1, 20 ± 1 and 40 ± 1°C for untreated erythrocytes. Our experiments with ozonized erythrocytes have shown a disappearance of the low temperature transition at 9°C. This means that ozone is able to eliminate the protein 4.1-dependent membrane thermal transition at 9 ± 1°C. The same situation was observed for chloropromazine-treated erythrocytes by Minetti & Di Stasi (1987). Ozone also exerts a dose-dependent influence on the high temperature transition at 40°C (Table 2). These results would indicate the involvement of cytoskeletal proteins as possible membrane target sites for ozone. This finding is in agreement with those reported in the literature (Wróbel *et al.*, 1997; Verweij *et al.*, 1982).

Wróbel and co-workers (1997) showed that structural changes in erythrocyte membranes treated with ozone may be mainly related to a specific modification caused by ozone in cytoskeletal proteins. Minetti *et al.* (1986), using circular dichroism and maleimide spin labelling in studies on purified spectrin, showed a slow thermal unfolding of the protein structure starting at around 30°C. Wróbel *et al.* (1997) suggest that in ozonized erythrocyte membranes the protein unfolding process seems to be more favourable. In fact, Verweij and co-workers (1982) showed that the treatment of spectrin with ozone resulted in covalent cross-linking of these proteins and led to the formation of *O,O′*-dityrosine.

Our experiments showed that treatment of erythrocytes with ozone at a concentration of 45 g/m^3 causes almost the same effects on the thermotropic transitions as those observed in Ca^{2+} treated cells. The effect of treatment of erythrocytes with the Ca^{2+} ionophore in the presence of 1.2 mM external calcium results in the disappearance of the low temperature break and the appearance of two breaks at 32°C and 15°C (Forte *et al.*, 1985). For ozonized erythrocytes we observed a gradual decrease of temperature transition following the increase of ozone concentration from 20°C (for controls) to 16°C for ozone concentration of 45 g/m^3. High transition temperature at 40°C observed for control erythrocytes after 45 g/m^3 ozone treatment was lowered to 37°C. Minetti *et al.* (1984) showed that the transition temperature occurring at 40°C in untreated erythrocytes after treatment with

anti-4.1 antibodies was lowered to 35°C. This would indicate that ozone at concentration 45 g/m^3 selectively affects band 4.1.

Treatment of erythrocytes with ozone at concentration of 10 g/m^3 results in increasing the high temperature transition from 40°C for untreated cells to 43°C, and to rigidization of cell membrane. Some observations suggest that cellular rigidity in oxidized erythrocytes can be associated with cross-linked membrane proteins, especially spectrin, forming high molecular mass complexes (Araiso *et al.*, 1986; Fung *et al.*, 1996). Aggregation of membrane proteins seems to be conducive to higher ordering of lipids and leads to an increase of thermotropic temperature transition. These dependences were observed both for lens cortical lipids and for erythrocyte membranes (Araiso *et al.*, 1986; Borchman *et al.*, 1993). Stearic acid spin-label freedom of motion is restricted by the spectrin–actin network and the proteins that link the cytoskeletal network to the membrane (Minetti & Di Stasi, 1987). Therefore, we suggest that the decrease of motion freedom of the spin probe 16-DSA in erythrocyte membranes treated with ozone at concentration of 10 g/m^3, observed in our experiments, is probably an effect of cross-linking of cytoskeletal proteins to high molecular aggregates. These protein aggregates can hinder motional freedom of the spin probe moiety.

It is well established that ozone reacts with membrane proteins, but it is not quite clear whether this leads to the cross-linking or degradation of proteins, or to both these processes simultaneously. Wróbel and co-workers (1997) suggest that medical doses of ozone cause a degradation rather than cross-linking of proteins. Ignatenko & Cherenkevich (1985) pointed out that ozone-induced *O,O′*-dityrosine formation could lead to both inter- and intramolecular cross-links. Gel filtration of ribonuclease treated with ozone ($5 \cdot 10^{-5}$ M) has not revealed the presence of high molecular fractions of this protein, indicating that, when the oxidation is not strong (with low doses of ozone), the destruction of peptide bonds of ribonuclease moiety is not effective. The low molecular forms of proteins in elution patterns, which testify to destruction of protein moiety, have been revealed only at the ozone concentrations higher than 10^{-4} M (corresponding to ozone concentration of 10 g/m^3) (Ignatenko & Cherenkevich, 1985). Our results from thermotropic transition experiments could suggest that ozonation of erythrocytes leads to structural changes in the membranes, especially in cytoskeletal proteins, but this effect is probably dose-dependent.

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