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Decrease in 2,2,6,6-tetramethyl-piperidine-1-oxyl (TEMPO) EPR signal in Ozone-treated Erythrocyte Membranes

ANNA WRÓBEL[a,*], ADAM JEZIERSKI[b] and JAN GOMUŁKIEWICZ[a]

[a]*Institute of Physics, Technical University of Wroclaw, Wybrzeże Wyspiańskiego 27, 50-370 Wroclaw, Poland;*
[b]*Faculty of Chemistry, University of Wroclaw, F. Joliot-Curie 14, 50-383 Wroclaw, Poland*

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In ozone-treated erythrocyte membrane suspension a slow decrease occurs in the EPR signal of 2,2,6,6-tetramethyl-piperidine-1-oxyl (TEMPO). Because of the absence of such a phenomenon in control membranes and ozonized buffer, this effect must be caused by reaction of nitroxide radicals with products of ozone reactions with membrane components. To find out which components are responsible for the decrease in EPR signal we studied this effect in simple model systems. The same phenomenon was observed both in lipid and protein systems treated by ozone. For unsaturated fatty acids, the correlation between the rate of decrease in EPR signal and the number of double bonds in the lipid molecule was very strong. This suggests that the observed decrease in the nitroxide radical TEMPO EPR signal in ozone-treated erythrocyte membranes is a complex process, but probably the most important reaction is recombination of nitroxide radicals with organic free radicals produced both in the process of lipid peroxidation and ozonolysis of double bonds.

Keywords: Nitroxide radical, ozone, electron paramagnetic resonance, erythrocyte membrane, oxidative stress

Abbreviations: TEMPO, 2,2,6,6-tetramethyl-piperidine-1-oxyl; PC, phosphatidylcholine; PE, phosphatidylethanolamine; BSA, bovine serum albumin; PBS, phosphate-buffered saline; 5-MSL, 3-maleimido-1-oxyl-2,2,5,5-tetramethylpyrrolidine; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid)

INTRODUCTION

The mechanism of ozone (O_3) toxicity is complex and the primary biochemical site of ozone reactivity responsible for cell destruction remains unknown. Membranes of cells and intracellular organelles may be the major sites of ozone damage.[1] The biological damage produced by ozone is attributable to its ability to cause an oxidative destruction of biomolecules either by a direct reaction or through the formation of free radicals and reactive intermediates, or by both processes.[2,3] Therefore, we conceptualize the damage caused by ozone in terms of a cascade of effects.[3] The reaction of ozone with membrane lipids occurs almost exclusively with the

*Corresponding author. E-mail: wrobel@rainbow.if.pwr.wroc.pl

carbon–carbon double bonds present in unsaturated fatty acids.[2,3] From various investigations, reaction pathways involving free radicals, hydroperoxides and ozonide formation have been proposed.[2–4] Ozone reacts with membrane proteins, particularly destroying cysteine, methionine, tryptophan and tyrosine residues.[5,6] To study the influence of ozone on biological membranes we chose the erythrocyte membrane. In our previous studies[7] concerning effect of ozone on fluidity of erythrocyte membranes we used nitroxide spin labels. Comparison of the electron paramagnetic resonance spectroscopy (EPR) parameters for control and ozone-treated samples shows that the ozonized membranes destroy the nitroxide spin labels. The significant decrease in EPR signal in ozonized membranes and lack of such a decrease in control membranes and those treated with ozonized buffer indicate that this process is caused by a reaction of nitroxide with products of ozone reactions with membrane components. To find out which membrane components are responsible for the decrease in EPR signal we studied this effect also in simple model systems using nitroxide radical TEMPO. It was found that a decrease in EPR signal in membranes is a complex process because the same was observed both in lipid and protein systems treated by ozone, like liposomes, and fatty acid and protein solution. For unsaturated fatty acids the correlation between the rate of decrease and the number of double bonds in lipid molecule was very strong, suggesting that the main role in the observed decrease in TEMPO EPR signal in ozone-treated membranes is played by a reaction of nitroxide radicals with organic free radicals produced both in the process of lipid peroxidation and ozonolysis of double bonds.

MATERIAL AND METHODS

Chemicals

TEMPO, PC from egg yolk, PC dimyristoyl (C 14 : 0), PE dimyristoyl (C 14 : 0), cholesterol, stearic acid, BSA, DTNB, GSH and GSSG were purchased from Sigma Chemical Co. Oleic acid and linolenic acid were obtained from Aldrich Chemical Co. Other reagents were of highest purity commercially available.

Erythrocyte Membrane Preparation

Bovine erythrocytes were isolated from fresh blood, anticoagulated with heparin (10 U/ml), by centrifugation at 4°C, at 2000× *g* and purified by three cycles of resuspension and washing with PBS (phosphate-buffered NaCl solution, 310 mosM, pH 7.4), after a careful removal of the buffy coat. The erythrocyte ghosts were prepared from the washed cells according to the method of Dodge *et al.*[8] The erythrocytes were hemolyzed on ice with 14 volumes of hypotonic phosphate buffer (20 mosM, pH 7.4) and centrifuged for 20 min at 4°C at 20 000× *g*. The ghosts were resuspended in ice-cold hypotonic phosphate buffer (20 mosM, pH 7.4), and this process was continued until the ghosts were free of residual hemoglobin. The protein concentration in the ghost suspension (of 4 mg/ml) was estimated by the method of Bradford[9] using bovine serum albumin as a standard. The -SH groups content was estimated using Ellman's reagent (DTNB).[10]

Liposome Preparation

Lipids were extracted from erythrocyte membranes by mixing one volume of an ice-cold washed erythrocyte suspension with 0.75 volume of ice-cold *n*-butanol.[11] The mixture was shaken at 0°C about 20 min. Then the butanol phase was separated by centrifugation at 0°C, at 22 000× *g*. After evaporation of *n*-butanol in a rotary evaporator, multilamellar liposomes were prepared by hydration of the lipid film in PBS.

Other Sample Preparation

Solutions of BSA, GSH, GSSG and suspensions of PC from egg yolk, PC dimyristoyl, PE

dimyristoyl, cholesterol, stearic acid, oleic acid and linolenic acid were prepared by mixing an appropriate amount of chemical with PBS buffer. The concentrations of all compounds are given in the figure captions.

Ozonation

Ozone was generated by passing pure gaseous oxygen through a Sorbios Ozone Generator model GSG 001.2 (Sorbios GmbH, Berlin, Germany) with flow rate of 10 l/h. The ozone concentration was controlled by the Ozone Analyzer BMT 961 (BMT Messtechnik GmbH, Berlin, Germany), an instrument using the direct UV-photometric method for the measurement of gaseous ozone with the measurement uncertainty of 1.5%. The ozone/oxygen mixture of concentration 45 g/m^3 of ozone in ozone/oxygen mixture (such a concentration is used in water disinfection and some medical applications) was passed for 2.5 min over a 10 ml volume of the stirred sample in PBS. Control samples were treated with pure oxygen and did not show any statistically significant differences compared to membranes treated with air (data not shown).

Electron Paramagnetic Resonance Spectroscopy

Twenty µl of TEMPO in water (1 mM) was added to one hundred µl of sample. After vigorous mixing the suspension was placed in glass haematocrit tubes (the height of samples in tube was 20 mm). Each tube containing a sample was sealed off and EPR spectrum was recorded at room temperature every 60 min for 8–10 h on X-band spectrometer, model SE/X 2543 (Radiopan, Poznań, Poland). The instrumental conditions were: field setting 334 mT, scan range of 10 mT, microwave power of 4 mW and modulation amplitude of 0.1 mT. The standard measurement conditions were typical for quantitative EPR (QEPR) technique. The height of the low field line (h_{+1}) of the TEMPO EPR signal was a measure of the relative signal intensity. Experiments were repeated for eight different blood samples and at least three times for each of model systems. The same effect was observed in all cases. Typical results of a single experiment have been collected in Figures 1–5 in order to demonstrate changes in TEMPO EPR signal intensity.

RESULTS

In ozone-treated erythrocyte membrane suspension there is a slow decrease in EPR signal of the nitroxide radical, TEMPO (Figure 1). Because of the absence of such a phenomenon in control membranes and ozonized buffer, this effect must be caused by a reaction of nitroxide radicals with products of ozone reactions with membrane components. The effect of ozone on the disappearance of TEMPO in simple lipid and protein systems is shown in Figures 2–5. We found that the reactions did not always show a simple first-order kinetics. Therefore, we have not calculated a rate constant. To check how ozone influences the decrease of the TEMPO signal in membranes

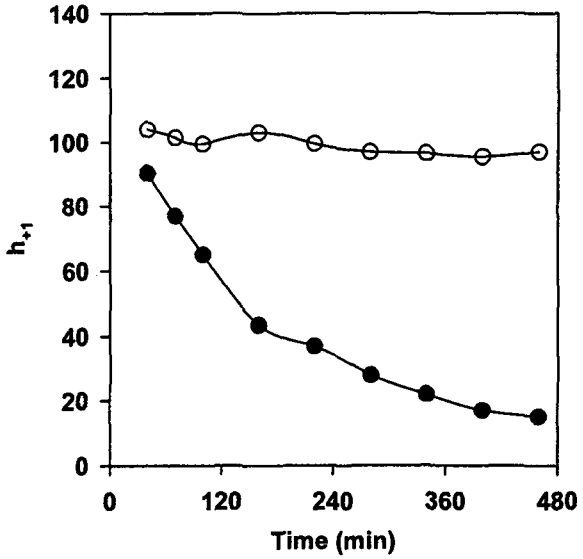

FIGURE 1 Amplitude of the EPR signal of TEMPO in control (○) and ozonized (●) erythrocyte membranes. (h_{+1}) – height of low field line (arbitrary units).

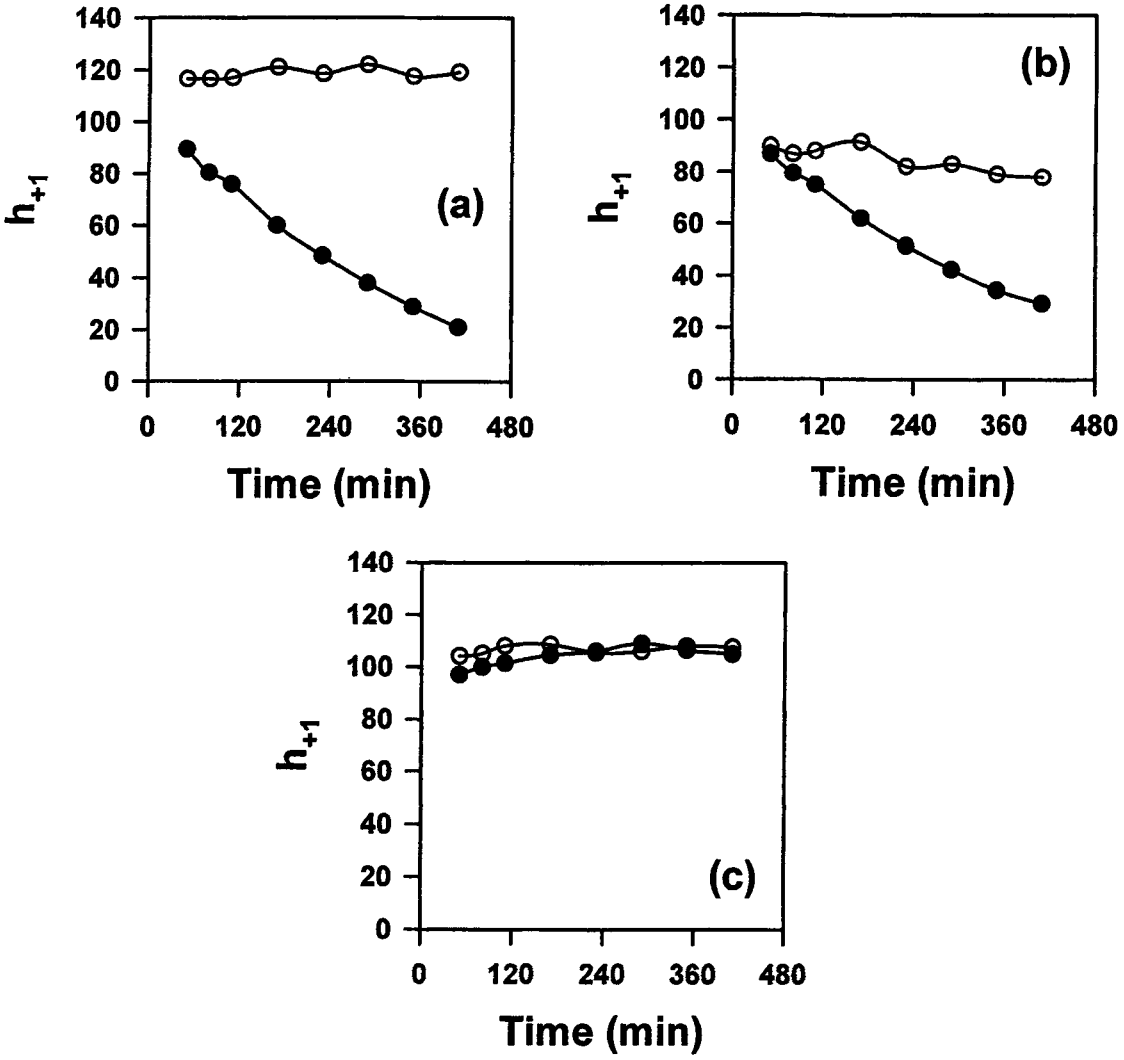

FIGURE 2 The amplitude of EPR signal of TEMPO in liposomes from extracted erythrocyte membrane lipids (a), PC from egg yolk (b) and PC 14:0 (c). Control (○) and ozonized (●) samples.

lacking proteins we conducted the experiments using liposomes prepared from extracted erythrocyte membrane lipids and from PC from egg yolk (Figure 2(a) and (b)). In both suspensions a slow decrease in EPR signal occurs, but in liposomes obtained from erythrocyte lipids the process seems to be faster. In contrast to these two systems consisting of natural lipids, in suspensions of synthetic saturated lipids PC (14 : 0) and PE (14 : 0) no decrease in EPR signal was observed (Figure 2(c)). Likewise, ozonation of stearic acid does not cause any decrease in TEMPO EPR signal. To find out if there existed any relationship between the number of double bonds in lipid molecule and the decrease in TEMPO EPR signal we have chosen two unsaturated fatty acids: oleic and linolenic. As shown in Figure 3 the amplitude of the TEMPO signal decreases much faster in the ozonized suspension of linolenic acid, with three double bonds in molecule, than in the suspension of oleic acid with only one double bond, and the rate of decrease is dependent on the concentration

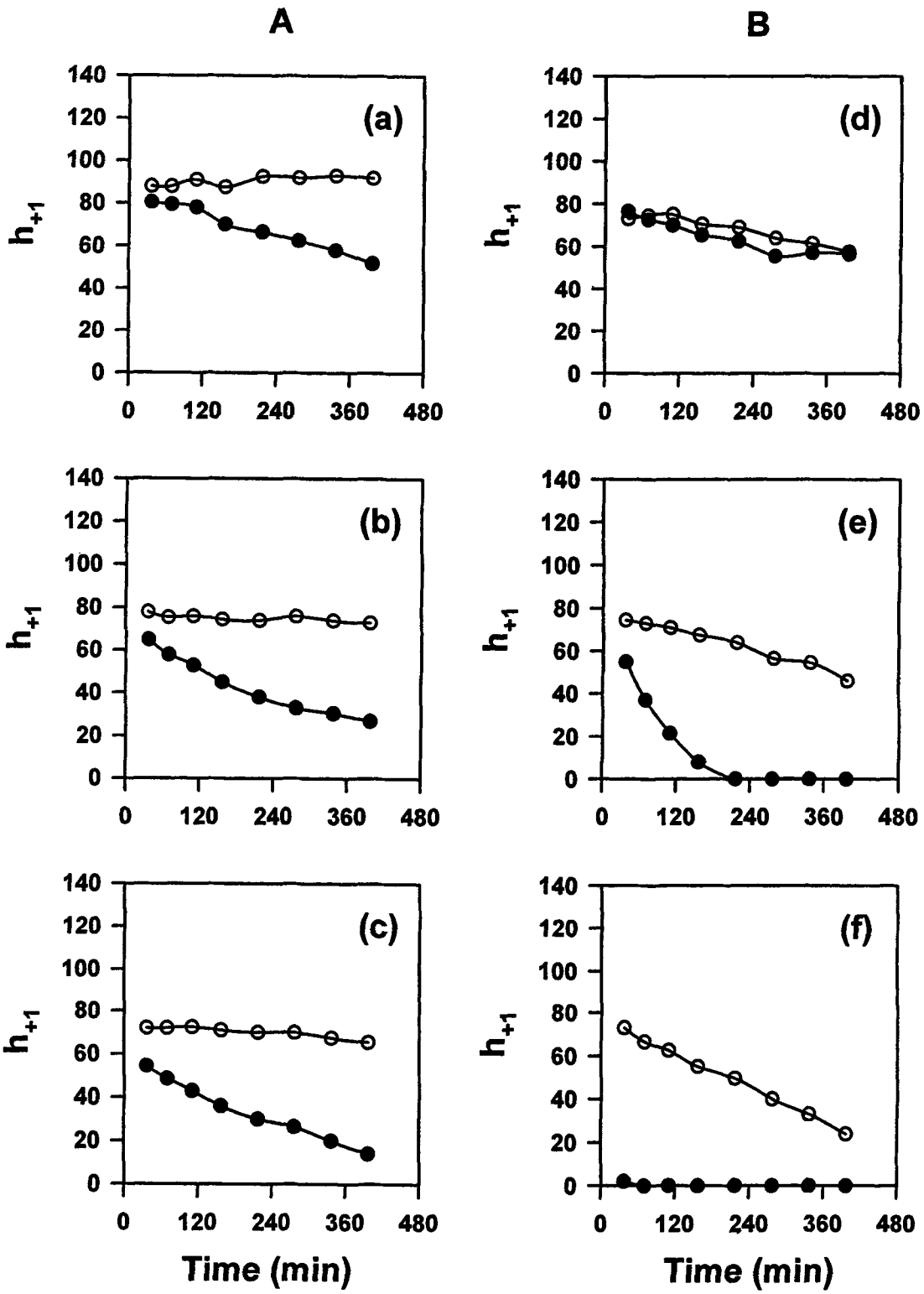

FIGURE 3 The amplitude of EPR signal of TEMPO in oleic (A) and linolenic (B) acid solution at concentration (mg/ml): 9.0 (a), 18.0 (b), 36.0 (c), 4.5 (d), 9.0 (e) and 18.0 (f). Control (○) and ozonized (●) samples.

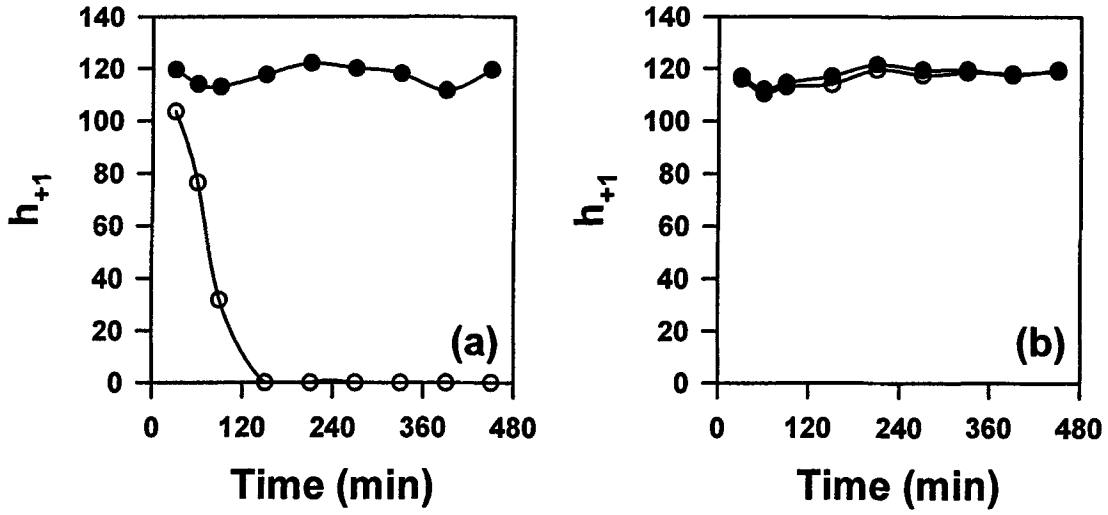

FIGURE 4 The amplitude of EPR signal of TEMPO in solutions of GSH (a) and GSSG (b) at concentration 0.64 mM. Control (○) and ozonized (●) samples.

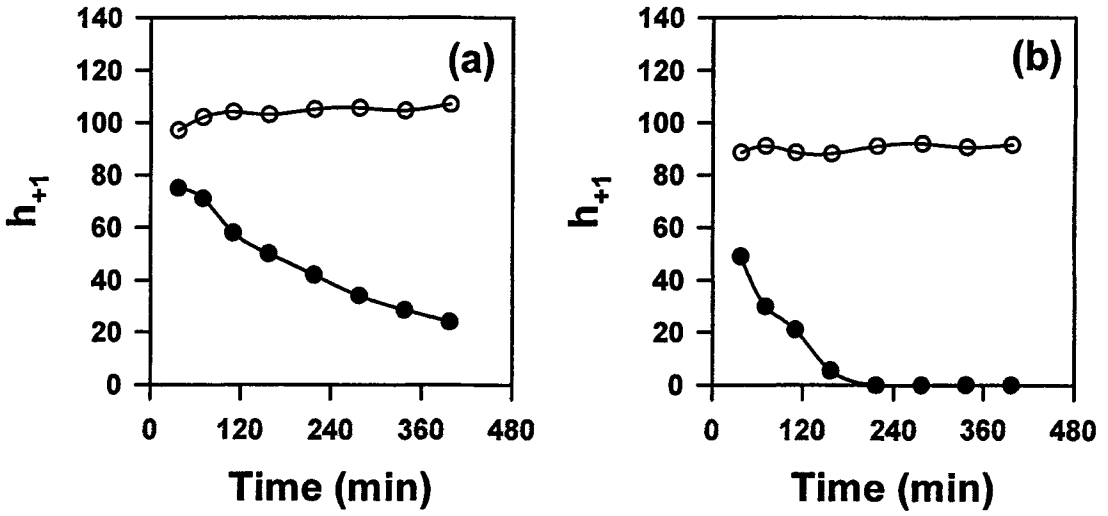

FIGURE 5 The amplitude of EPR signal of TEMPO in bovine serum albumin at concentration (mg/ml): 4.0 (a) and 8.0 (b). Control (○) and ozonized (●) samples.

of fatty acid (compare Figure 3A-a with B-e and A-b with B-f). In contrast to the suspension of oleic acid, in the control suspension of linolenic acid a decrease in the signal was also observed. This effect may be caused by a reaction of TEMPO with products of lipid peroxidation on air. Ozonation of cholesterol, one of the main components of many animal membranes, does not cause any decrease in the TEMPO signal amplitude.

Usually in biological systems the most important process resulting in a loss of the paramagnetism of nitroxide radicals is reduction. The basic source of reducing factors in biological membranes may be -SH groups of proteins. With GSH, the decrease in EPR signal is observed only in control samples and is not observed after ozonation (Figure 4(a)) when all -SH groups are oxidized. GSSG does not cause any decrease in

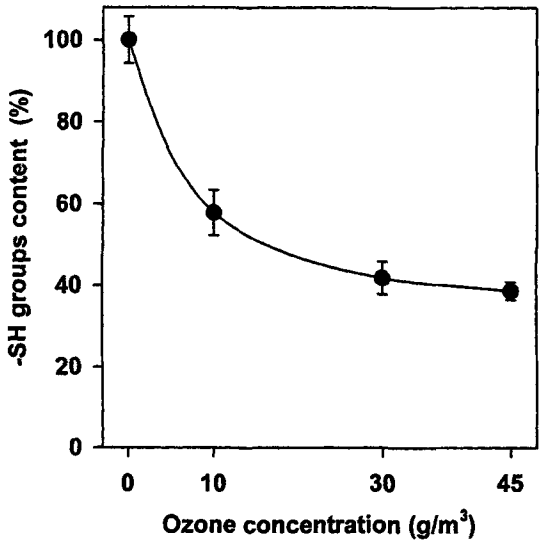

FIGURE 6 Ozone-induced decrease in -SH content in bovine erythrocyte membranes. Each point in the figure is an average of 7 different blood samples. The error bars show SD.

the signal in both control and ozonized samples (Figure 4(b)). The control solution of BSA at a concentration comparable to protein concentration in erythrocyte membranes does not cause any decrease in EPR signal but ozonation of BSA solution led to a concentration-dependent decrease in TEMPO amplitude (Figure 5). Since in ozonized membranes the level of -SH groups is significantly decreased (Figure 6), facts described above indicate, that reduction of nitroxide by -SH groups of proteins does not play the main role in the loss of EPR signal after ozonation.

DISCUSSION

The use of nitroxide radicals in living systems has greatly expanded recently. Nitroxide components, including TEMPO, have been used classically as probes for membrane structure[12] and sensors of oxygen in biological systems.[13] Over the past few years nitroxides have been shown to possess antioxidant activity and protect cells against a variety of agents that impose oxidative stress.[14–16] The main reactions of nitroxide radicals resulting in a loss of the paramagnetism are oxidation, reduction and free radical recombination.[17] Usually in biological systems the main reaction is reduction. The basic source of reducing factors in erythrocyte membranes are -SH groups of proteins. A good example of the decrease in TEMPO EPR signal caused by a reaction with -SH groups is the reaction with GSH (Figure 4(a)). Oxidation of GSH by ozone completely inhibits this process. Because in membranes treated with ozone the level of -SH groups is lowered by about 50% (Figure 6) the observed loss of TEMPO EPR signal in ozonized membranes cannot be caused by a reduction of nitroxides by -SH groups. However, reaction of ozone with -SH groups may produce superoxides.[18] The presence of a superoxide in ozonized membranes is possible, since we have observed an increase in h_w/h_s ratio derived from EPR spectra of maleimide spin label 5-MSL incorporated into membranes.[7] h_w/h_s is the ratio of signal amplitudes of weakly and strongly immobilized spin label residues and this ratio was shown to be elevated in erythrocyte membranes treated with superoxide.[19] In contrast, this ratio decreases in membranes treated with hydroxyl radicals.[19] It was found that superoxide can reduce nitroxide free radicals to their corresponding hydroxylamines in the presence of -SH containing compounds.[20] The apparent rate constant for reduction is two orders of magnitude larger than the rate constant for oxidation of hydroxylamine by superoxide.[21] Because in membranes treated with ozone the level of -SH groups is about 50% compared to control sample the reduction of nitroxide by superoxide in the presence of -SH groups cannot be excluded. The same process may take place also in the loss of EPR signal in BSA solution. Nitroxide radicals were reported to act as SOD mimics and catalyze the dismutation of superoxide radicals.[14,17] However, in this reaction the nitroxide concentration is practically unaffected. This means that the process does not play the main role in the loss of EPR signal in ozonized membranes.

The observed loss of the TEMPO EPR signal in model lipid systems suggests that this process is related to the reaction of ozone with double bonds in the lipid molecule. Ozone reacts with double bonds by two principal mechanisms: one is the classical Criegee mechanism to give Criegee ozonide, and aldehydes and hydrogen peroxide if water is present;[2,3] the second possibility is to react by a mechanism producing free radicals.[2,4] The fraction of ozone reacting to form free radicals is not larger than 1%, but if these ozone-produced free radicals initiate lipid peroxidation, then they have the potential of producing significant damage. Both carbon- and oxygen-centered radicals were detected by the EPR spin trapping method in ozone-treated fatty acids emulsions and PC liposomes.[4] TEMPO reacts readily with alkyl radicals, and slowly with peroxl radicals.[17] The decay in the TEMPO EPR signal in the ozone-treated erythrocyte membranes and fatty acid suspensions observed by us may be caused by the recombination of nitroxide radicals with free organic radicals produced both in the process of lipid peroxidation and ozonolysis of double bonds. Because monounsaturated fatty acids such as oleic acid do not undergo autoxidation, the observed loss of EPR signal in oleic acid suspension (Figure 3A) is not caused by a reaction of nitroxide radicals with products of lipid peroxidation. In suspension of linolenic acid peroxidation of lipids may occur. This is evidenced by the decrease in EPR signal observed also in control samples (Figure 3B). This effect does not occur in a control suspension of oleic acid (Figure 3A). Concentration-dependent loss of EPR signal in control suspension of linolenic acid is probably caused by a reaction of nitroxide radicals with products of lipid peroxidation arising from contact of linolenic acid with atmospheric oxygen. Because lipids in erythrocyte membrane have fatty acid residues with different number of double bonds, the decrease in EPR signal observed in ozonized membranes may be caused by a reaction of nitroxide with products of lipid peroxidation. However, it was shown using oleic acid, that the loss of the EPR signal may occur also when lipid peroxidation does not take place.

Membrane proteins located in the immediate vicinity of lipids may also be the target of radicals, in particular those derived from lipid peroxidation and also by products of Criegee ozonation. If water is present, ozonation should yield 1 mol of hydrogen peroxide and 2 mol of aldehyde for each mole of ozone and mole of unsaturated fatty acid that reacts.[2,3] The reaction of hydrogen peroxide with proteins may lead to the formation of protein free radicals detected by EPR spectroscopy.[22–24] The superhyperfine couplings derived from EPR spectra of spin traps indicate that in most cases the radical site was located on the ring of tyrosine[23,24] or tryptophan.[22] Recombination of protein radicals with nitroxide radicals may play an important role in the observed decrease in EPR signal of TEMPO in erythrocyte membranes and BSA solution. Formation of protein radicals was found in systems that undergo various oxidative stresses, such as gamma irradiation,[25] porphyrin-sensitized photo-oxidation,[26] peroxynitrite[27] and hydrogen peroxide treatment.[22–24] Oxidized proteins are often functionally inactive and their unfolding in association with enhanced susceptibility to proteinases[28] may be the primary target responsible for cellular damage.

Our previous studies on the effect of ozone on fluidity of erythrocyte membranes indicate much smaller changes in the lipid phase of the ozonized membranes, compared to those shown in membrane proteins.[7] This fact seems to confirm the thesis, that in the reaction of ozone with the cell membrane the proteins play a significant role.

The loss of EPR signal of nitroxide radicals in suspension of membranes undergoing oxidative stress caused by ozone, observed by us may be related to novel applications of nitroxides as antioxidants. Nitroxides have been shown to protect cells against a variety of agents that impose oxidative stress by inhibition of lipid peroxidation as preventive and chain-breaking antioxidants,[16,29] by function as superoxide

dismutase mimics[14] and protection DNA.[15] The protective activity of various nitroxides against oxidative damage to lipids has been well established in literature.[16,29] Comparatively fewer papers touch on protective effect of nitroxides to proteins.[30]

In conclusion, we postulate that the decrease in TEMPO EPR signal in ozone-treated erythrocyte membranes may be explained by three main mechanisms:

1. Recombination of nitroxides with free radicals resulting from ozonation of alkenes. This mechanism is clearly demonstrated by comparing the rate of decrease in TEMPO amplitude in fatty acids with different number of double bonds in the molecule.
2. Reduction of nitroxide to its corresponding hydroxylamine resulting from the simultaneous presence of superoxide, thiol and nitroxide. This mechanism may lead to the clearance of TEMPO in ozonized membranes and protein solution.
3. Recombination of nitroxides with peroxyl radicals of proteins, which may be responsible for the decrease in TEMPO amplitude in membrane suspension and protein solution.

Certainly, our observations do not enable an estimate of contribution of each of these processes to the loss of TEMPO in ozonized membranes, but they indicate that the antioxidant activity of nitroxide radicals is a complex phenomenon and the role of other molecules than lipids, especially proteins, is also important.

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