

Received 25 July 2001
Accepted 23 October 2001

**DECREASE IN 2,2,6,6-TETRAMETHYL-PIPERIDINE-1-OXYL
(TEMPO) EPR SIGNAL IN PEROXYNITRITE-TREATED
ERYTHROCYTE MEMBRANES**

ANNA WRÓBEL

Institute of Physics, Wrocław University of Technology,
Wybrzeże Wyspiańskiego 27, 50-370 Wrocław, Poland

Abstract: The treatment of erythrocyte membranes with peroxynitrite (ONOO^-), a cytotoxic species formed *in vivo* by the almost completely diffusion controlled reaction of nitric oxide (NO^\bullet) and the superoxide anion ($\text{O}_2^{\bullet-}$), led to the loss of the EPR signal of the nitroxide radical 2,2,6,6-tetramethyl-piperidine-1-oxyl (TEMPO). The decrease in the TEMPO EPR signal was peroxynitrite concentration dependent in the studied peroxynitrite concentration range (100 – 1000 μM). The absence of such a phenomenon in the control membranes (not treated with peroxynitrite) and in a buffer treated with peroxynitrite indicates that the effect must be caused by nitroxide radicals reacting with the products of peroxynitrite reactions with membrane components. To find out which membrane components are responsible for the decrease in EPR signal, this effect was studied in simple model systems (protein and lipid suspensions). The same phenomenon was observed in both lipid and protein systems treated with peroxynitrite, but in protein solutions the effect was greater and occurred for lower peroxynitrite concentrations. A clear effect of the loss of the EPR signal was observed for both erythrocyte membranes and bovine serum albumin (BSA) solution for a peroxynitrite concentration of 100 μM , while in the case of linolenic acid suspension, a significant difference between control and peroxynitrite-treated samples was achieved for a peroxynitrite concentration of 1000 μM . A comparison of the results obtained for the lipid and protein systems suggests that the reaction of nitroxide radicals

e-mail: wrobel@rainbow.if.pwr.wroc.pl, fax: (+4871) 3283696

Abbreviations: TEMPO, 2,2,6,6-tetramethyl-piperidine-1-oxyl; BSA, bovine serum albumin; PBS, phosphate buffered saline; EDTA, ethylenediaminetetraacetate.

with protein derived species plays the main role in the observed decrease in the TEMPO EPR signal in peroxynitrite treated erythrocyte membranes.

Key Words: Peroxynitrite, Nitroxide Radical TEMPO, Electron Paramagnetic Resonance, Erythrocyte Membrane, Oxidative Stress

INTRODUCTION

Peroxynitrite (ONOO^-) is formed by the almost completely diffusion controlled reaction of nitric oxide (NO^\bullet) and the superoxide anion ($\text{O}_2^{\bullet-}$) [1], and its formation *in vivo* probably occurs near such cells as macrophages, neutrophils and endothelial cells that produce both nitric oxide and superoxide [2-5]. This compound is a potent and versatile oxidizing and nitrating agent, and several lines of evidence point to peroxynitrite as a key biomolecule in mediating the reactivity and toxicity of nitric oxide and superoxide, the molecules of which are relatively unreactive towards most organic molecules [6, 7]. ONOO^- is a stable anion in alkaline solution (pK 6.8); however, upon protonation, ONOOH decays rapidly, with a half-life of 1s at pH 7.4 [1], generating reactive species that readily react with biomolecules including sulfhydryls [8-10], lipids [5, 7, 11] and amino acids [2, 12-14]. This wide range of biological targets makes peroxynitrite a potent tissue-damaging species implicated in several pathological situations like inflammatory disorders [2, 5, 15], atherosclerosis [6] or neurodegenerative diseases such as Alzheimer's disease [16, 17].

The mechanism of peroxynitrite toxicity is complex, and the primary biochemical site of peroxynitrite reactivity responsible for cell destruction remains unknown. Membranes of cells and intracellular organelles may be the sites of peroxynitrite damage [7, 11, 18, 19]. The biological damage produced by peroxynitrite in membranes is attributable to its ability to destroy biomolecules either by a direct reaction or through the formation of free radicals and reactive intermediates, or via both processes [7, 11, 20]. The reaction of peroxynitrite with membrane lipids results in the production of lipid hydroperoxides [11]. It was found that lipid peroxidation is the main channel of interaction of lipids and peroxynitrite in the presence of oxygen [7]. This implies that there is a mechanism whereby peroxynitrite induces lipid free radicals formation [21]. Peroxynitrite reaction with proteins can nitrate as well as oxidize amino acid residues and affect prosthetic groups [2, 12-14]. The most reactive amino acids are cysteine, methionine, tryptophan and tyrosine [12, 13]. Peroxynitrite-mediated oxidation and nitration of amino acids proceeds through both one- and two- electron mechanisms, the former leading to the formation of protein radicals [13, 14, 22, 23].

To study the influence of peroxynitrite on biological membranes, I chose the erythrocyte membranes, a simple and suitable model system often used for studies of plasma membrane modifications by toxic agents. By means of

electron paramagnetic spectroscopy, a significant decrease in the EPR signal of the nitroxide radical TEMPO was observed in peroxyxynitrite-treated erythrocyte membranes. The lack of such a phenomenon in the control membranes (not treated with peroxyxynitrite), in those membranes treated with the products of peroxyxynitrite decomposition in buffer, and in buffer treated with peroxyxynitrite indicates that the process is caused by nitroxides reacting with the products of peroxyxynitrite reactions with membrane components. The results of studies on the same effect in simple lipid and protein model systems suggest that the main role in the observed decrease in the TEMPO EPR signal in peroxyxynitrite treated membranes is played by nitroxide radicals reacting with protein derived species, probably free radicals.

MATERIALS AND METHODS

Chemicals

TEMPO and BSA were purchased from Sigma Chemical Co. Oleic acid and linolenic acid were obtained from Aldrich Chemical Co. HBS cellulose was from Serva Chemical Co.

Erythrocyte membranes preparation

Bovine erythrocytes were isolated from fresh blood, anticoagulated with citrate, 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). The osmolarity was calculated by totalling the concentration of all ionizable species. After careful removal of the buffy coat, residual leukocytes were removed by passing the erythrocyte suspension through a column of HBS cellulose. Erythrocyte ghosts were prepared from the washed cells according to a modification of the method of Dodge *et al.* [24]. The erythrocytes were hemolysed on ice with 14 volumes of hypotonic phosphate buffer (20 mosM, pH 7.4), containing 1 mM EDTA as a proteolytic inhibitor, and centrifuged for 20 min at 4°C at 20000×g. The ghosts were resuspended in ice-cold hypotonic phosphate buffer (20 mosM, pH 7.4) with EDTA, and this process was continued until the ghosts were free of residual hemoglobin. The ghosts were resuspended in phosphate buffer without EDTA, and the protein concentration in the suspension (of 4 mg/ml) was estimated by the method of Bradford [25] using bovine serum albumin as a standard.

Other samples preparation

Solutions of BSA were prepared by mixing an appropriate amount of the protein with PBS buffer. Suspensions of oleic acid and linolenic acid were prepared in 0.1 M sodium carbonate (the pH of these samples was 8.5). The concentrations of all the compounds are given in the figure captions.

Peroxynitrite synthesis

Peroxynitrite was synthesized by azide-ozone reaction [26]. This method provides concentrated solutions of peroxynitrite that are low in ionic strength, low in alkali and free of H₂O₂. An ozone stream generated by a Sorbios Ozone Generator model GSG 001.2 (Sorbios GmbH, Berlin, Germany) was bubbled through a glass-frit into 100 ml of 0.1 M sodium azide in water (pH adjusted previously to 12 with 1 N NaOH) chilled to 0°C in an ice water mixture for about 60 min., with a flow rate of 40 l/h and an ozone concentration of 20 g/m³. Peroxynitrite formation was monitored spectrophotometrically during the synthesis at 302 nm ($\epsilon = 1670 \text{ M}^{-1} \text{ cm}^{-1}$). The synthesis was stopped about 10 min after obtaining the maximum concentration. The final concentration of peroxynitrite was about 45 mM. The stock solution was stored at -18°C and used within 1 week of synthesis. Before each experiment the concentration of peroxynitrite was estimated spectrophotometrically [26] and an appropriate volume of stock solution was added to the sample. Control samples were treated with water (pH 12). To check for the potential effect of the products of peroxynitrite decomposition, peroxynitrite was allowed to decompose for 24 h at 37°C, before mixing with the samples.

Electron paramagnetic resonance spectroscopy

Ten μl of TEMPO in water ($c=0.4 \text{ mM}$) was added to two hundred μl of sample that had been treated with peroxynitrite. After vigorous mixing, the suspension was placed in glass haematocrit tubes (the height of samples in the tubes was 20 mm). Each tube containing a sample was sealed off, and the EPR spectrum was recorded at room temperature for 2 to 6 hours in an X-band spectrometer, model SE/X 2543 (Radiopan, Poznań, Poland). The instrumental conditions were: field setting 334 mT, scan range 10 mT, microwave power 4 mW and modulation amplitude 0.1 mT. The standard measurement conditions were typical for the 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 seven different blood samples and at least three times for each model system. The same effect was observed in all cases. The typical results of a single experiment have been summarized in Figures 1-3 in order to demonstrate changes in TEMPO EPR signal intensity.

RESULTS

In the peroxynitrite-treated erythrocyte membrane suspension, there is a slow decrease in the EPR signal of the nitroxide radical TEMPO (Fig. 1). Although the majority of the decrease in the TEMPO signal occurs before the first EPR measurement, direct interaction between TEMPO and peroxynitrite has been excluded by the lack of the decay in TEMPO EPR signal in peroxynitrite treated

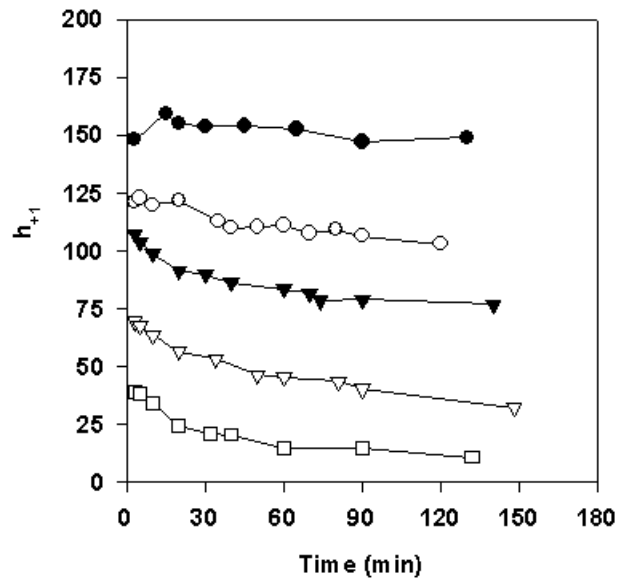


Fig.1. The amplitude of the EPR signal of TEMPO in control (■) and peroxynitrite-treated erythrocyte membranes. Peroxynitrite concentration (μM): 100 (□), 250 (△), 500 (▽) and 1000 (○). (h_{+1}) - height of low field line (arbitrary units).

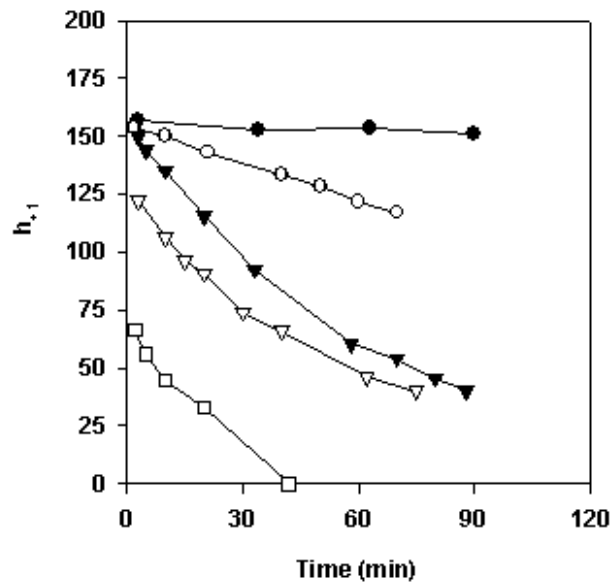


Fig. 2. The amplitude of the EPR signal of TEMPO in control (■) and peroxynitrite-treated bovine serum albumin at 8 mg/ml concentration. Peroxynitrite concentration (μM): 100 (□), 250 (△), 500 (▽) and 1000 (○).

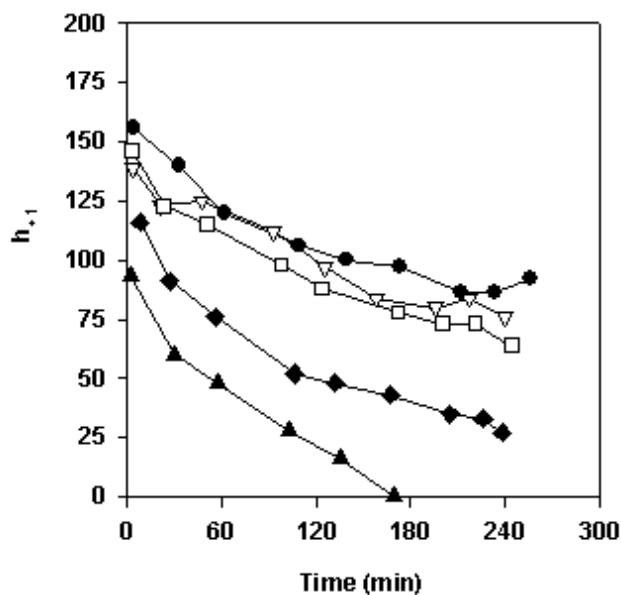


Fig. 3. The amplitude of the EPR signal of TEMPO in control (I) and peroxynitrite-treated linolenic acid suspension of 36 mg/ml concentration. Peroxynitrite concentration (μM): 500 (∇), 1000 (\circ), 2000 (\blacklozenge) and 3000 (\blacktriangle).

buffer. Because of the absence of a decrease in TEMPO signal amplitude in the control membranes, in the membranes treated with the products of peroxynitrite decomposition, and in buffer treated with peroxynitrite, the effect must be caused by nitroxide radicals reacting with the products of peroxynitrite reactions with membrane components. A study of this effect in simple lipid and protein model systems showed that a decrease in the EPR signal in the membrane is a complex process, because the same phenomenon was observed both in lipid and protein solutions (Fig. 2 and 3).

Peroxynitrite treatment of BSA solution led to a concentration-dependent decrease in TEMPO signal amplitude (Fig. 2). A clear effect of the loss of EPR signal was observed both for erythrocyte membranes and BSA solution for low peroxynitrite concentrations (100 μM), but in BSA solution this process had quite a different course. In peroxynitrite treated erythrocyte membranes, the rapid decrease in TEMPO signal amplitude takes place within the first 3 min of the addition of nitroxide to the sample (Fig. 1). In peroxynitrite BSA solution, there was no rapid phase decrease immediately after TEMPO addition (except for the highest concentration of peroxynitrite – 1000 μM), and the signal decreased slowly dependent on peroxynitrite concentration.

To find out if the same process takes place in lipids, two unsaturated fatty acids with different number of double bonds were chosen: oleic and linolenic.

Peroxynitrite treatment of oleic acid does not cause any decrease in the TEMPO EPR signal (data not shown). In the case of peroxynitrite treated linolenic acid, the decrease in nitroxide concentration was clear, but to get results comparable to those obtained for erythrocyte membrane suspension and BSA solution, a much higher peroxynitrite concentration had to be used (compare curves for 1000 μM peroxynitrite from Fig. 1, 2 and 3). The loss of the TEMPO signal in the control suspension of linolenic acid was probably caused by the reaction of TEMPO with the products of lipid peroxidation on air.

DISCUSSION

To better understand the mechanism of peroxynitrite cytotoxicity and its role in pathological processes, it is important to characterize its reactivity toward different biomolecules. Studying the effect of peroxynitrite on the cell membrane may help to understand the role played by oxidation damage in the pathogenesis of some human diseases including neurodegenerative disorders, and to find effective defenses against the excessive production of this compound. The loss of the EPR signal of nitroxide radicals in erythrocyte membranes treated with peroxynitrite observed in this study may be related to novel applications of nitroxides as antioxidants. Over the past few years, nitroxides have been shown to possess antioxidant activity and to protect cells against a variety of agents that impose oxidative stress [27-29]. It was found that nitroxide 4-hydroxy TEMPO inhibits peroxynitrite luminol-dependent chemiluminescence (LDCL), is a potential tool to control peroxynitrite mediated toxicity in activated microglia cell in culture [30], and significantly reduces the formation of peroxynitrite in rats subjected to carrageenan-induced pleurisy [15]. A greater knowledge of the interaction between nitroxides and peroxynitrite in biological systems will aid in the design of rational therapies for pharmacological intervention using nitroxides.

My studies show that in peroxynitrite treated erythrocyte membranes, there is a slow decrease in the EPR signal of the nitroxide radical TEMPO. Because ONOOH decays rapidly after protonation at pH 7.4 [1], this phenomenon cannot be the result of the reaction of peroxynitrite with nitroxides, but must be caused by nitroxide radicals reacting with products of peroxynitrite reaction with membrane components. The main reactions of nitroxide radicals resulting in a loss of paramagnetism are oxidation, reduction and free radical recombination. Usually, in biological systems the main reaction of nitroxides is reduction. The basic source of reducing factors in erythrocyte membranes are the -SH groups of proteins. Peroxynitrite mediates the oxidation of both nonprotein and protein -SH groups [8-10, 18]. In erythrocyte membranes, peroxynitrite dependent decrease in protein -SH groups is significant (50% at 250 μM peroxynitrite) [18]. This means that the observed loss of the TEMPO EPR signal in peroxynitrite treated membranes cannot be caused by the

reduction of nitroxides by -SH groups. However, peroxynitrite may initiate the autoxidation of -SH groups, a process known to produce superoxide [31]. Superoxide can reduce nitroxide free radicals to their corresponding hydroxylamines in the presence of -SH containing compounds [32]. The apparent rate constant for reduction is two orders of magnitude larger than the rate constant for oxidation of hydroxylamine by superoxide [33]. Because in membranes treated with peroxynitrite not all -SH groups are oxidized, the reduction of nitroxide by superoxide in the presence of -SH groups cannot be excluded. The same process may also take place in the case of loss of the EPR signal in BSA solution, because after peroxynitrite treatment, non-oxidized -SH groups remain [9].

Peroxynitrite oxidation of proteins occurs, at least partially, through one-electron-transfer oxidative processes - EPR spin-trapping studies of the peroxynitrite-mediated oxidation of albumin demonstrated that the corresponding thiyl radicals were formed [13, 22]. In the reaction of peroxynitrite with BSA, the most reactive amino acids are cysteine, methionine and tryptophan [12, 13]. The amino acids that react slowly with peroxynitrite could possibly still have been modified by reaction with highly reactive species formed from peroxynitrite in rate limiting steps. The reactions of these species with tyrosine are likely to lead to tyrosine nitration [12]. It was found that in the reaction of peroxynitrite with human blood plasma, tyrosine- and tryptophan-centered radicals are formed [13, 23]. This peroxynitrite-mediated one electron oxidation of biomolecules may be an important event in its cytotoxic mechanism. Protein radicals formation was found in systems that undergo various oxidative stresses, such as gamma irradiation [34], porphyrin-sensitized photo-oxidation [35], and hydrogen peroxide treatment [36]. Oxidized proteins are often functionally inactive and their unfolding in association with enhanced susceptibility to proteinases may be the primary target responsible for cellular damage. Recombination of protein radicals produced by peroxynitrite with nitroxide radicals may play an important role in the observed decrease in the EPR signal of TEMPO in erythrocyte membranes and BSA solution.

In peroxynitrite treated lipid suspension, a slow decrease in nitroxide concentration was also observed. the reaction of peroxynitrite with lipids initiates lipid peroxidation [7, 11, 21]. Peroxynitrite addition to phosphatidylcholine liposomes resulted in MDA and conjugated diene formation, as well as oxygen consumption [7]. It was also shown that peroxynitrite causes the production of phospholipid hydroperoxides in synaptosomes [11]. The last theoretical study supports the hypothesis that the reactive species in lipid peroxidation with peroxynitrous acid in the presence of air is the discrete hydroxyl radical [21], which is formed by homolysis of the O-O bond in this acid. Peroxynitrous acid is generated from the peroxynitrite anion at physiological pH. Lipid free radicals formed during the lipid peroxidation process of linolenic acid may recombine with nitroxide radicals

causing the loss of the EPR signal. Because monounsaturated fatty acids such as oleic acid do not undergo autoxidation, the lack of a loss of the EPR signal in peroxynitrite treated oleic acid suspension may confirm this hypothesis.

In this study I tried to compare the decrease in the EPR signal of nitroxide radical TEMPO in peroxynitrite treated protein and lipid systems. It was found that proteins are probably the main target of peroxynitrite reaction. A clear effect of the loss of the EPR signal was observed both for erythrocyte membranes and BSA solution for a low peroxynitrite concentration (100 μM), while in the case of linolenic acid, a significant difference between the control and peroxynitrite treated samples was observed for a peroxynitrite concentration of 1000 μM , and total loss of the EPR signal was achieved for a peroxynitrite concentration of 3000 μM . The fact that in linolenic acid samples (which have higher pH than other samples, which means a longer half-life of peroxynitrite) the effect is much smaller, additionally confirming the thesis that peroxynitrite reacts much more easily with proteins than lipids. The observations are in accordance with studies concerning peroxynitrite induced hemolysis of human erythrocytes [19]. It was shown that erythrocytes underwent hemolysis when incubated with peroxynitrite without appreciable lipid peroxidation [19]. The studies on mouse erythrocyte membranes indicate that the cytoskeleton, in particular spectrin, is a very sensitive target of peroxynitrite [18] and studies on human erythrocytes show the formation of erythrocyte membrane protein aggregates, and a decrease in the content of many protein bands (mostly spectrin and band 3 protein) [37]. Peroxynitrite was also demonstrated to inhibit multidrug resistance-associated protein (MRP) in erythrocyte [38] and synaptic plasma membrane Ca^{2+} -ATPase [39]. These facts seem to confirm the thesis that in the reaction of peroxynitrite with the cell membrane, proteins play a significant role.

In conclusion, this study enables an estimate of the contribution of processes linked to lipids and proteins to the loss of TEMPO in peroxynitrite treated membranes. I postulate that the decrease in the TEMPO EPR signal in peroxynitrite-treated erythrocyte membranes may be explained mostly by the recombination of nitroxides with free radicals produced by the reaction of peroxynitrite with membrane proteins, or less likely with free radicals derived from the process of lipid peroxidation. The fact that in our previous studies on the effect of ozone on erythrocyte membranes the difference in decrease of the TEMPO EPR signal between protein and lipid systems was much less significant [40] indicates that the reactions of nitroxide radicals with the products of membrane component oxidation strongly depend on the oxidant. This must be considered in novel applications of nitroxides as tools protecting cells against agents that impose oxidative stress.

Acknowledgements. I am grateful to Ms Agata Dryjańska for her excellent technical assistance.

REFERENCES

1. Beckman, J.S., Beckman T.W., Chen, J., Marshall, P.A. and Freeman B.A. Apparent hydroxyl radical production by peroxynitrite: Implications for endothelial injury from nitric oxide and superoxide. **Proc. Natl. Acad. Sci. USA** 87 (1990) 1620-1624.
2. MacMillan-Crow, L.A., Crow, J.P., Kerby, J.D., Beckman, J.S. and Thomson, J.A. Nitration and inactivation of manganese superoxide dismutase in chronic rejection of human renal allografts. **Proc. Natl. Acad. Sci. USA** 93 (1996) 11853-11858.
3. Rosen, G.M. and Freeman, B.A. Detection of superoxide generated by endothelial cells. **Proc. Natl. Acad. Sci. USA** 81 (1984) 7269-7273.
4. Palmer, R.M.J., Ferrige, A.G. and Moncada, S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. **Nature** 327 (1987) 524-526.
5. Rodenas, J., Carbonell, T. and Mitjavila, M.T. Different roles for nitrogen monoxide and peroxynitrite in lipid peroxidation induced by activated neutrophils. **Free Radic. Biol. Med.** 28 (2000) 374-380.
6. White, C.R., Brock, T.A., Chang, L.Y., Crapo, J., Briscoe, P., Ku, D., Bradley, W.A., Gianturio, S.H., Gore, J., Freeman, B.A. and Tarpey, M.M. Superoxide and peroxynitrite in atherosclerosis. **Proc. Natl. Acad. Sci. USA** 91 (1994) 1044-1048.
7. Radi, R., Beckman, J.S., Bush, K.M. and Freeman, B.A. Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide. **Arch. Biochem. Biophys.** 288 (1991) 481- 487.
8. Whiteman, M. and Halliwell, B. Thiols and disulphide can aggravate peroxynitrite-dependent inactivation of α_1 -antiproteinase. **FEBS Lett.** 414 (1997) 497-500.
9. Radi, R., Beckman, J.S., Bush, K.M. and Freeman, B.A. Peroxynitrite oxidation of sulfhydryls. **J. Biol. Chem.** 266 (1991) 4244-4250.
10. Quijano, C., Alvarez, B., Gatti, R.M., Augusto, O. And. Radi, R. Pathways of peroxynitrite oxidation of thiols groups. **Biochem. J.** 322 (1997) 167-173.
11. Shi, H., Noguchi, N., Xu, Y. and Niki, E. Formation of phospholipid hydroperoxides and its inhibition by α -tocopherol in rat brain synaptosomes induced by peroxynitrite. **Biochem. Biophys. Res. Commun.** 257 (1999) 651-656.
12. Alvarez, B., Ferrer-Süeta, G., Freeman, B.A. and Radi, R. Kinetics of peroxynitrite reaction with amino acids and human serum albumin. **J. Biol. Chem.** 274 (1999) 842-848.
13. Pietraforte, D. and Minetti, M. Direct ESR detection of peroxynitrite-induced tyrosine-centred protein radicals in human blood plasma. **Biochem. J.** 325 (1997) 675-684.

14. Minetti, M., Scorza, G. and Pietraforte, D. Peroxynitrite induces long-lived tyrosyl radical(s) in oxyhemoglobin of red blood cells through a reaction involving CO₂ and ferryl species. **Biochemistry** 38 (1999) 2078-2087.
15. Cuzzocrea, S., McDonald, M.C., Mota Filipe, H., Costantino, G., Mazzon, E., Santagati, S., Caputi, A.P. and Thiemermann, C. Effects of tempol, a membrane-permeable radical scavenger, in a rodent model of carrageenan-induced pleurisy. **Eur. J. Pharmacol.** 390 (2000) 209-222.
16. Lipton, S.A., Chou, Y.-B., Pan, Z.-H., Lei, S.Z., Chen, H.-S.V., Sucher, N.J., Loscaizo, J., Singel, D.J. and Stamler, J. S. (1993) A redox-based mechanism for the neuroprotective and neurodestructive effects of nitric oxide and related nitroso-compounds. **Nature** 364 (1993) 626- 632.
17. Pappolla, M. A., Chyan, Y.-J., Poeggeler, B., Frangione, B., Wilson, G., Ghiso, J. and Reiter, R.J. An assessment of the antioxidant and the antiamyloidogenic properties of melatonin: implications for Alzheimer's disease. **J. Neural Transm.** 107 (2000) 203-231.
18. Di Mascio, P., Dewez, B. and Garcia, C. R. S. Ghost protein damage by peroxynitrite and its protection by melatonin. **Braz. J. Med. Biol. Res.** 33 (2000) 11-17.
19. Kondo, H., Takahashi, M. and Niki, E. Peroxynitrite-induced hemolysis of human erythrocytes and its inhibition by antioxidants. **FEBS Lett.** 413 (1997) 236-238.
20. Karoui, H., Hogg, N., Frjaviile, C., Tordos, P. and Kalyanaraman, B. **J.Biol. Chem.** 271 (1996) 6000-6009.
21. Shustov, G. V., Spinney, R. and Rauk, A. Mechanism of peroxynitrite oxidation of aliphatic CH bonds in saturated and unsaturated hydrocarbons. A theoretical model for the CH oxidation of lipids. **J. Am. Chem. Soc.** 122 (2000) 1191-199.
22. Vasquez-Vivar, J., Santos, A. M., Junquera, V. B. C. and Augusto, O. Peroxynitrite-mediated formation of free radicals in human plasma: EPR detection of ascorbyl, albumin-thiyl and uric acid-derived free radicals. **Biochem. J.** 314 (1996) 869-876.
23. Pietraforte, D. and Minetti, M. One-electron oxidation pathway of peroxynitrite decomposition in human blood plasma: evidence for the formation of protein tryptophan-centred radicals. **Biochem. J.** 321 (1997) 743-750.
24. Dodge, J. T., Mitchell, C. and Hanahan, D. J. The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. **Arch. Biochem. Biophys.** 100 (1963) 119-130.
25. Bradford, M.A. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. **Anal. Biochem.** 72 (1976) 248-254.

26. Uppu, R. M., Squadrito, G. L., Cueto R, and Pryor, W. A. Synthesis of peroxynitrite by azide-ozone reaction. **Meth. Enzymol.** 269 (1996), 311-321.
27. Mitchell, J. B., Samuni, A., Krishna, M. C., deGraff, W. G., Ahn, M. S., Samuni, U. and Russo, A. Biologically active metal-independent superoxide dismutase mimics. **Biochemistry** 29(1990) 2802-2807.
28. Villarini, M., Moretti, M., Damiani, E., Greci, L., Santroni, A. M., Fedeli, D. and Falcioni, G. Detection of DNA damage in stressed trout nucleated erythrocytes using the comet assay-protection by nitroxide radicals. **Free Radic. Biol. Med.** 24 (1998) 310-1315.
29. Samuni, A. M. and Barenholz, Y. Stable nitroxide radicals protect lipid acyl chains from radiation damage. **Free Radic. Biol. Med.** 22 (1997) 1165-1174.
30. Van Dyke, K., Sacks, M., Birkle, D. and Reasor, M. 4-hydroxy-tempo: a potential tool to control peroxynitrite-mediated toxicity. **FASEB J.** 12 (1998) 4421-4421.
31. Lemerrier, J-N., Squadrito, G. L. and Pryor, W. A. Spin trap studies on the decomposition of peroxynitrite. **Arch. Biochem. Biophys.** 321 (1995) 31-39.
32. Finkelstein, E., Rosen, G. M. and Rauckman, E. J. Superoxide-dependent reduction of nitroxides by thiols. **Biochim. Biophys. Acta** 802 (1984) 90-98.
33. Finkelstein, E., Rosen, G. M. and Rauckman, E. J. Spin trapping of superoxide and hydroxyl radical: practical aspects. **Arch. Biochem. Biophys.** 200 (1980) 1-16.
34. Miyazaki, T., Yoshimura, T., Mita, K., Suzuki, K. and Watanabe, M. Rate constant for reaction of vitamin C with protein radicals in gamma irradiated aqueous albumin solution at 295 K. **Radiat. Phys. Chem.** 45 (1995) 199-202.
35. Silvester, J. A., Timmins, G. S. and Davies, M. J. Photodynamically generated bovine serum albumin radicals - evidence for damage transfer and oxidation at cysteine and tryptophan residues. **Free Radic. Biol. Med.** 24 (1998) 754-766.
36. Gunther, M. R., Tschirretguth, R. A., Witkowska, H. E., Fann, Y. C., Barr, D. P., Demontellano, P. R. O. and Mason, R. P. Site-specific spin-trapping of tyrosine radicals in the oxidation of metmyoglobin by hydrogen peroxide. **Biochem. J.** 330 (1998) 1293-1299.
37. Soszyński, M. and Bartosz, G. Effects of peroxynitrite on erythrocytes. **Biochim. Biophys. Acta** 1291 (1996) 107-114.
38. Soszyński, M. and Bartosz, G. Peroxynitrite inhibits glutathione S-conjugate transport. **Biochim. Biophys. Acta** 1325 (1997) 135-141.

39. Zadi, A. and Michaelis, M.L. Effects of reactive oxygen species on brain synaptic plasma membrane Ca^{2+} -ATPase. **Free Radic. Biol. Med.** 27 (1999) 810-821.
40. Wróbel, A., Jezierski, A. and Gomul'kiewicz, J. Decrease in 2,2,6,6-tetramethyl-piperidine-1-oxyl (TEMPO) EPR signal in ozone-treated erythrocyte membranes. **Free Rad. Res.** 31 (1999) 201-210.