Free Rad. Res., Vol. 28, pp. 507–516
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The Effect of Indolinic and Quinolinic Nitroxide Radicals on Trout Erythrocytes Exposed to Oxidative Stress

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Accepted by Prof. E. Niki

(Received 20 July 1997; In Revised form 24 October 1997)

The purpose of this study was to evaluate the ability of indolinic and quinolinic nitroxide radicals to protect trout (*Salmo irideus*) erythrocytes against oxidative stress. By using laurdan as a fluorescence probe, it was observed that the nitroxides inhibited the shift towards a gel phase of liposomes prepared with phospholipids extracted from trout erythrocyte membranes prior to the hemolytic event. In addition, the presence of 100 μM nitroxides in these liposomes protected the latter against lipid peroxidation determined by monitoring conjugated diene formation. However, the short chain analogue of the indolinic nitroxide and the quinolinic nitroxide had a negative effect on trout hemolysis, contrary to what has already been observed in previous studies on human RBCs (red blood cells). The half-time ($t_{1/2}$) of the hemolytic process was 174 ± 4.02 min for the former and 184 ± 4.30 min for the latter compared to the control, 283 ± 5.05 min. Furthermore, the nitroxides remarkably increased the autoxidation rate of both trout and human hemoglobin to met-Hb. Even though protection at the membrane level is conferred by the nitroxides during the early stages of lipid peroxidation, their antioxidative ability might be overwhelmed at a later stage by other mechanisms such as the increased autoxidation of hemoglobin in the presence of the nitroxides, thus giving a possible explanation for the early induction of hemolysis induced by the nitroxides. The superoxide scavenging ability of all the nitroxides used was also evaluated through chemiluminescence.

Keywords: Indolinic and quinolinic nitroxides, superoxide radical, trout erythrocytes, hemolysis, fluorescence, conjugated dienes

Abbreviations: CL, chemiluminescence; Hb, hemoglobin; GP, generalized polarization; RBCs, red blood cells; TBARS, thiobarbituric acid reactive substances

INTRODUCTION

An imbalance in the oxidant/antioxidant status of the cell is associated with oxidative stress, and

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this determines a cascade of events in the cell, such as lipid peroxidation and protein oxidation, which ultimately lead to the loss of structural and functional integrity at the membrane level.[1,2] The primary defence system against ROS (reactive oxygen species) is constituted by the enzymes superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and further supported by metal binding proteins such as transferrin, albumin, caeruloplasmin, haptoglobin. The secondary defence system is provided by vitamins E, C, β-carotenes as well as glutathione, urates, bilirubin and others which all have scavenging properties against an overproduction of free radicals that may incidentally occur during a cell's life cycle.[3]

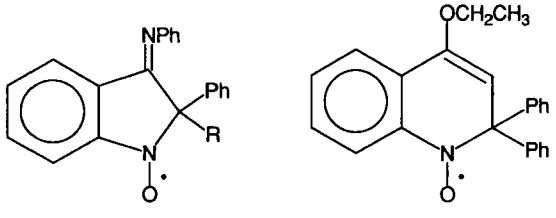
In this last decade, a great deal of research has been devoted to the study of different types of antioxidants which may somehow mimic our natural endogenous ones or at least minimize the deleterious effects produced by ROS.[4] Our interest in this field has focused on stable nitroxide compounds which have been recently used in biological systems for their capacity to mimic superoxide dismutase.[5–8] Two different catalytic pathways have been proposed for the dismutation of superoxide radical by various piperidinyl and pyrrolidine nitroxide radicals. The first one suggests that the catalytic cycle involves the reduced form of the nitroxide radical i.e. the hydroxylamine,[9] while in the second one, the intermediate species involved is the oxoammonium ion which is the oxidized form of the nitroxide.[10] These mechanisms obviously depend upon the redox potentials of the nitroxides considered compared to the redox potential of the couple $O_2^{\bullet -}/H_2O_2$. Other mechanisms could also be involved in the superoxide dismutation by nitroxide radicals[11] and this subject is still under study.

In the present study, a different class of nitroxide radicals, namely indolinic **1–3** and quinolinic nitroxides **4** were subjected to a flux of superoxide radical generated by the system xanthine/xanthine oxidase to see whether they were capable of scavenging this species. This is the first in the cascade of ROS to be produced by cellular metabolism in an oxygen-containing environment and it is detoxified by superoxide dismutase. The scavenging of superoxide radical by these compounds was studied by chemiluminescence. In addition, the eventual antioxidant capacity of these four nitroxides was followed in a biological system consisting of trout erythrocytes exposed to oxidative stress induced thermally and by a variation in pH. These nucleated cells in a relatively short time at 35°C and pH 6.3 undergo hemolysis; this phenomenon is in part both due to the formation of superoxide radical and to the inactivation of glutathione peroxidase which are a consequence of Hb oxidation as reported previously.[12–14] For these reasons, trout erythrocytes are excellent tools for studying the effects of oxidative stress at the membrane level in a relatively short time. The influence of the nitroxides on oxidation of trout erythrocytes was evaluated by different means. Firstly, the extent of hemolysis in the presence of the nitroxides was assessed. Subsequently, a fluorescence study concerning with the physical–chemical modifications at the membrane level was carried out. In this case, laurdan (2-dimethylamino-(6-lauroyl)-naphthalene) was selected to probe the changes in membrane polarity before the hemolytic event. This probe incorporates at the hydrophilic–hydrophobic interface of the membrane with the lauric acid tail anchored in the hydrophobic region of the bilayer. The probe displays spectral sensitivity to the polarity of its surroundings which is related to the physical state and dynamics of the surrounding phospholipid polar head group. Measurement of conjugated dienes was also performed in order to evaluate the extent of lipid peroxidation of erythrocytic membranes in the presence of the nitroxide radicals. Since hemoglobin plays an important role on membrane modifications during oxidative stress, the effect exerted by the nitroxides on the degree of Hb oxidation was also investigated.

MATERIALS AND METHODS

All reagents were of pure analytical grade. Xanthine, xanthine oxidase (E.C. 1.1.3.22) and lucigenin were purchased from Sigma Chem. Co., St. Louis Mo (USA). Laurdan was purchased from Molecular Probes (Eugene, OR). Nitroxides **1** (1,2-dihydro-2-ethyl-2-phenyl-3H-indole-3-phenylimino-1-oxyl), **2** (1,2-dihydro-2-decyl-2-phenyl-3H-indole-3-phenylimino-1-oxyl), **3** (1,2-dihydro-2-octadecyl-2-phenyl-3H-indole-3-phenylimino-1-oxyl) and **4** (1,2-dihydro-2,2-diphenyl-4-ethoxy-quinoline-1-oxyl) were respectively prepared according to the literature methods (Scheme 1).[15–17]

Chemiluminescence measurements were performed using lucigenin as chemiluminogenic probe, and superoxide radical was produced by the xanthine/xanthine oxidase system. Chemiluminescence (CL) was measured in an Autolumat LB 953 (Berthol Co. Wildbad, Germany) in a reaction mixture containing 0.9 U/ml xanthine oxidase, 150 μM lucigenin in physiological solution and different concentrations of nitroxide compounds dissolved in ethanol (maximum volume 1%). The reaction was started by injecting xanthine at a final concentration of 50 μM and followed for 60 s as described previously.[18] The values are expressed as counts per second (cps).



1 R=CH_2CH_3
2 R=$(CH_2)_9CH_3$
3 R=$(CH_2)_{17}CH_3$

4

SCHEME 1 Nitroxide **1**: 1,2-dihydro-2-ethyl-2-phenyl-3H-indole-3-phenylimino-1-oxyl; Nitroxide **2**: 1,2-dihydro-2-decyl-2-phenyl-3H-indole-3-phenylimino-1-oxyl; Nitroxide **3**: 1,2-dihydro-2-octadecyl-2-phenyl-3H-indole-3-phenylimino-1-oxyl; Nitroxide **4**: 1,2-dihydro-2,2-diphenyl-4-ethoxy-quinoline-1-oxyl.

RBCs were obtained from *Salmo irideus*, an inbred strain of trout. Blood was extracted by puncturing the lateral tail vein. After washing, the cells were suspended in isotonic medium (0.1 M phosphate buffer, 0.1 M NaCl, 0.2% citrate, 1 mM EDTA) at pH 6.3 and incubated at 35°C. The degree of hemolysis was determined as previously described[19] either in the presence or in the absence of the desired amount of nitroxide dissolved in ethanol. In particular, this was determined as $(100 \times A/10 \times A^*100\%)$, where A is the optical density of Hb present in the supernatant after centrifugation of red cell suspension, and $A^*100\%$ is the optical density of the red cell suspension after complete lysis with 10 volumes distilled water at zero time incubation. Experiments involving carbonmonoxyhemoglobin were carried out after exposure of the red blood cell suspension to a weak vacuum and then to pure CO gas.[19] All controls were carried out with the same concentration of ethanol used in the samples containing nitroxide compounds.

The fluorescence and "oxidation index" experiments were performed on liposomes obtained using phospholipids extracted from nucleus-free erythrocyte membranes at the beginning (0′) and after 1 h of incubation of the RBC suspension, before the hemolytic event. Nucleus-free erythrocyte membranes were prepared according to Steer and Levitzki[20] using a discontinuous sucrose gradient. All samples were normalized by Lowry's method.[21] Liposomes were prepared according to the method of Folch.[22]

Steady-state fluorescence on the phospholipids extracted from erythrocyte membranes was performed on an HITACHI 4500 spectrofluorometer. Generalized polarization of laurdan, described by Parasassi *et al.*[23] was calculated by exciting laurdan at 340 nm (GP_{340}) according to the following equation:

$$GP = I_B - I_R/I_B + I_R$$

where I_B and I_R are the emission intensities at the blue (440 nm) and red (490 nm) edges of the

emission spectrum and correspond to the fluorescence emission maxima in the gel and liquid-crystalline phases, respectively of the bilayer.[24] The final probe concentration was 1 μM and fluorescence measurements were performed at 21°C.

The absorbance ratio $A_{233\,nm}/A_{215\,nm}$, defined as the "oxidation index" was used as a relative measurement for conjugated dienes which are formed during the early stages of lipid peroxidation, according to the method introduced by Konings.[25]

Hemoglobin A and trout hemoglobin components were obtained as previously described.[26,27] Hemoglobin (1 mg/ml) was suspended in phosphate buffer 0.1 M, pH 7.5 and incubated at 35°C in the presence of 20 μM nitroxides dissolved in ethanol. The rate of met-Hb formation was followed in a Cary 219 spectrophotometer in the visible region; reference values (i.e. complete reduction and oxidation) were estimated by addition of sodium dithionite and ferricyanide, respectively.

RESULTS

In this study, scavenging of superoxide radical by nitroxides **1–4** was evaluated by the CL technique. Chemiluminescence determinations were performed by using lucigenin as chemiluminogenic probe for superoxide radical produced by xanthine/xanthine oxidase.[28] The reaction of lucigenin with superoxide radical, gives rise to chemiluminescence and the level of CL indicates the presence of superoxide in the medium under study.[29,30] In order to confirm that the origin of CL observed was due to superoxide radical, superoxide dismutase was added in the reaction mixture and 100% inhibition of the CL signal was obtained with about 20 μg/ml of superoxide dismutase. Moreover, the addition of catalase (1000 U/ml) or inactivated superoxide dismutase (20 μg/ml) did not significantly alter the CL value (results not shown). In order to rule out a possible interaction between nitroxides and lucigenin EPR experiments were performed in the same conditions as in the chemiluminescence assay. No decrease in the EPR signal of the nitroxides was observed when lucigenin was added to a solution of the nitroxide (data not shown). Figure 1 shows the kinetics of the lucigenin amplified chemiluminescence measured in the presence of indolinic nitroxides (**1–3**) which differ in the length of the acyl side chain, being $-CH_2-CH_3$, $-(CH_2)_9-CH_3$ and $-(CH_2)_{17}-CH_3$ the respective chains of nitroxides **1**, **2** and **3**, and in the presence of quinolinic nitroxide **4**. The data obtained with a fixed concentration (100 μM) show that all these nitroxides are capable of scavenging superoxide radical and this decreases in the following order:

1 > 4 > 3 > 2.

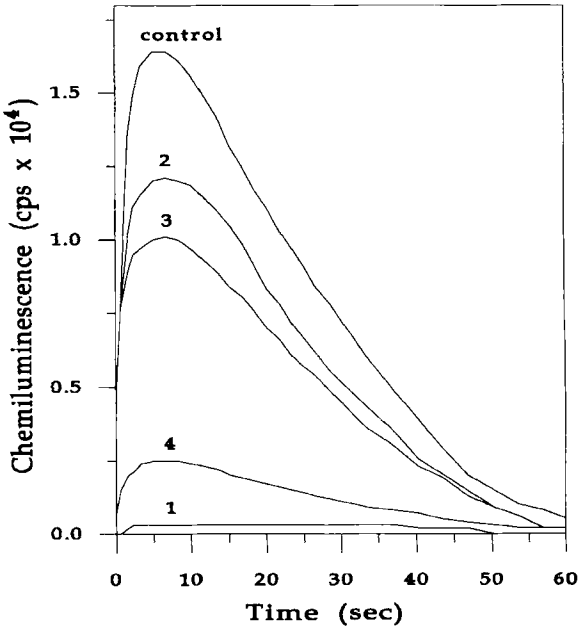


FIGURE 1 Kinetics of the lucigenin-amplified CL measured in the presence and in the absence of indolinic and quinolinic nitroxides. CL was measured in the presence of 0.9 U/ml xanthine oxidase, 150 μM lucigenin and 100 μM of indolinic and quinolinic nitroxides; the reaction was started by injection of xanthine at a final concentration of 50 μM in physiological solution. Chemiluminescence was measured as counts per seconds (cps). Decay constant calculated from the curves expressed as $t_{1/2}$ are the following: control, 26.40 s; **2**, 25.20 s; **3**, 27.00 s; **4**, 28.80 s; **1**, 32.40 s.

The maximum value of CL was 1.595×10^4 cps for the control in the presence of the same volume of ethanol as that used for the nitroxide samples. The other values were respectively 4.333×10^2 cps for nitroxide **1**, 1.248×10^4 cps for nitroxide **2** and 1.023×10^4 cps for nitroxide **3**. As can be observed in the same figure, quinolinic nitroxide **4** at the same concentration of 100 μM gave a maximum peak of 2.555×10^3. The area under the curves are as follows: control, 107.9 cm^2; **2**, 78.3 cm^2; **3**, 68.7 cm^2; **4**, 18.1 cm^2; **1**, 0.4 cm^2. No substantial differences in the decay constants expressed as $t_{1/2}$ were observed between the nitroxides.

The influence of these compounds on the rate of hemolysis in trout erythrocytes after incubation in isotonic buffer at 35°C and pH 6.3 was also investigated. In Table I, the half-time ($t_{1/2}$) of hemolysis of a suspension containing 2×10^6 RBCs/ml in the presence and in the absence of a fixed concentration (100 μM) of nitroxides **1–4** is reported. The half-time expresses the time necessary for 50% hemolysis to occur and it was determined from Figure 2 which shows the time course of hemolysis. It is possible to observe that all the nitroxides have a negative effect on the rate of hemolysis. It is necessary to

TABLE I Influence of different nitroxides on hemolysis of trout erythrocyte suspensions. Conditions: after washing, cells (2×10^6 RBCs/ml) were suspended in isotonic medium at pH 6.3 and incubated at 35°C in the presence and in the absence of 100 μM of nitroxides. See Materials and Methods. Results are mean ± s.d. $n = 3$

Sample	Hemolysis (half-time in min)
Control	283 ± 5.05
Control + Ethanol	285 ± 5.32
1	174 ± 4.02
2	244 ± 6.04
3	250 ± 5.20
4	184 ± 4.30

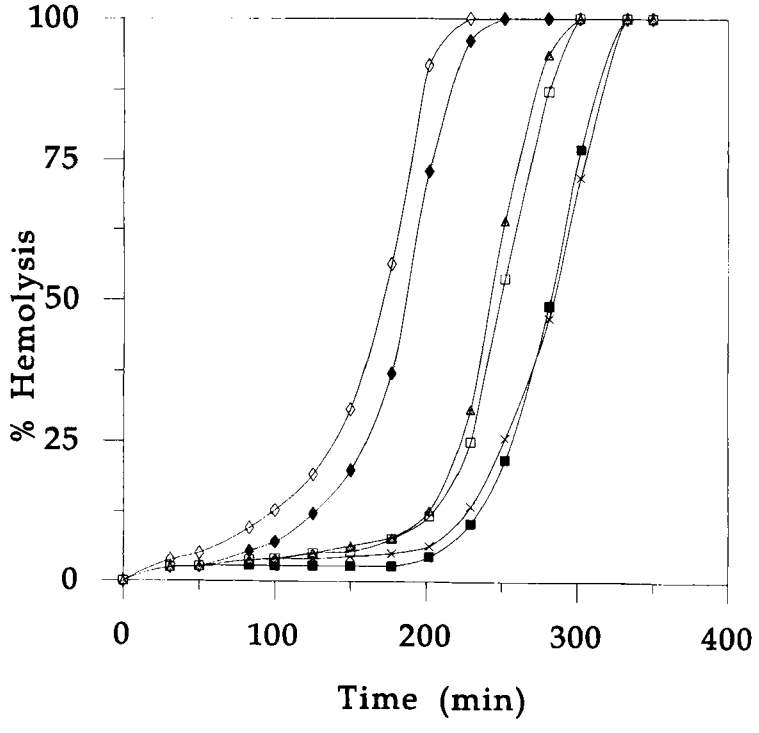


FIGURE 2 Time course of hemolysis of trout erythrocyte suspensions in the presence of different nitroxides. Conditions: after washing, cells (2×10^6 RBCs/ml) were suspended in isotonic medium at pH 6.3 and incubated at 35°C in the presence and in the absence of 100 μM of nitroxides. See Materials and Methods. Control = ■; control with ethanol = ×; nitroxide **1** = ◇; nitroxide **2** = ▲; nitroxide **3** = □; nitroxide **4** = ◆.

point out that trout erythrocyte hemolysis occurs in the above conditions independently of the presence of nitroxides. However, their presence accelerates this phenomenon. The susceptibility to hemolysis in the presence of nitroxides **2** and **3** is the same whilst it is enhanced in the presence of nitroxide **1** and **4**. Similar experiments were performed in the presence of carbon monoxide; in this case, hemolysis in the presence of the nitroxides was reduced by about 60% (data not reported).

The results on fluorescence experiments of generalized polarization for laurdan embedded in liposomes consisting of lipids extracted from erythrocyte membranes both in the presence and absence of nitroxides **1** and **4** are shown in Table II. The samples were analyzed both at 0′ and after 1 h of incubation. This one hour time frame was established since up to this time the extent of hemolysis was irrelevant. Nitroxides **1** and **4** were chosen as these were the two compounds which exerted a strong influence on the rate of hemolysis (Table I) and on the capacity to scavenge superoxide radical (Figure 1). The data obtained show a significant ($p = 0$) increase in GP_{340} values in the absence of the nitroxides after 1 h of incubation. However, in the presence of **1** and **4** after the same incubation time, there is no significant decrease in the GP_{340} value.

Table III shows the "oxidation index" of lipids extracted from trout erythrocyte membranes obtained from the above mentioned samples. In this case, an increase in conjugated diene formation for the control sample is present after 1 h of incubation; after the same time frame no increase in conjugated diene formation is observed in those samples incubated in the presence of nitroxides **1** and **4**.

In a previous study[31] where modified human erythrocytes were used, it was observed that the presence of nitroxides **1** and **4** retarded the hemolytic event, contrary to what is observed in this present study. Therefore, in order to better understand the reasons underlying erythrocyte hemolysis in the presence of nitroxide radicals, a series of experiments were undertaken to assess the possible influence that these nitroxides could have on the stability of both, human and trout hemoglobins. It is known that trout erythrocytes contain four hemoglobin components[32] called HbI, HbII, HbIII and HbIV. Two of these, HbI and HbIV, represent, respectively, about 20% and 60% of the whole pigment and have very different oxygen-binding properties. In Table IV, the half-time ($t_{1/2}$) of hemoglobin autoxidation for three different hemoproteins (HbA, HbI and HbIV) incubated at 35°C, is reported. The three hemoglobins have a different autoxidation rate. The half-time ($t_{1/2}$) of this process corresponds to about 465 min of incubation for HbA while for HbI and HbIV it is 180 and 198 min respectively. Nitroxides **2** and **3** do not influence the hemoglobin oxidation rate. However, both compounds **1** and **4** increase the autoxidation rate of the three hemoglobins tested.

TABLE II Influence of nitroxides **1** and **4** on Generalized Polarization of laurdan (GP_{340}) in liposomes made of phospholipids extracted from trout erythrocyte membranes. Conditions: the erythrocytes were suspended in isotonic buffer pH 6.3 and incubated for 1 h at 35°C in presence of 100 μM of nitroxides. See Materials and Methods. Data represent the mean ± s.d. $n = 3$

Time (h)	GP_{340}		
	Control	**1**	**4**
0	0.236 ± 0.018	0.235 ± 0.096	0.232 ± 0.039
1	0.311 ± 0.013	0.283 ± 0.003	0.245 ± 0.004

TABLE III Influence of nitroxides **1** and **4** on conjugated diene formation. The oxidation index is obtained by the absorbance ratio $A_{233\,nm}/A_{215\,nm}$ measured on liposomes prepared with phospholipids extracted from trout RBCs, in the absence and in the presence of 100 μM nitroxides at the beginning (0′) and after 1 h of incubation of the RBCs suspension. See Materials and Methods. Results are mean ± s.d. $n = 3$

Time (h)	$A_{233\,nm}/A_{215\,nm}$		
	Control	**1**	**4**
0	0.768 ± 0.031	0.877 ± 0.135	0.921 ± 0.073
1	0.979 ± 0.003	0.714 ± 0.025	0.742 ± 0.002

TABLE IV Influence of 20 μM nitroxides **1–4** on the rate of autoxidation of human HbA, trout HbI and trout HbIV. See Materials and Methods. Results are mean ± s.d. $n = 3$

Sample	Autoxidation (half-time in min)				
	Control	**1**	**2**	**3**	**4**
HbA	465 ± 15.05	72.5 ± 2.40	475 ± 15.37	480 ± 15.53	137.5 ± 4.55
HbI	180 ± 10.20	22.5 ± 1.27	195 ± 11.05	190 ± 10.76	55.0 ± 3.11
HbIV	198 ± 19.09	15.0 ± 1.50	196 ± 18.90	200 ± 19.30	47.5 ± 4.55

All the results presented are representative of at least three experiments and appropriate controls were carried out throughout.

DISCUSSION

Nitroxide radicals have long been used as biophysical markers to probe cellular components and metabolism[33] and as nuclear magnetic resonance imaging contrast agents.[34] It is only recently that their antioxidative activity in biological systems has been investigated and exploited.[35] However, the majority of studies and literature reports deal with piperidine, pyrrolidine and oxazolidine nitroxide derivatives.[5–8] Here, we discuss the superoxide scavenging ability of an aromatic class of nitroxides, namely indolinic (**1–3**) and quinolinic (**4**) and their effect on trout erythrocytes exposed to oxidative stress.

The inhibition of superoxide-induced chemiluminescence by nitroxides **1–4** can be clearly observed in Figure 1. This result was not surprising since these nitroxides react with a wide range of oxygen-centered radicals such as peroxyl[36] and alkoxyl[37] radicals yielding non-paramagnetic products of the nitroxide, as well as with carbon-centered radicals[38] giving rise to the corresponding alkylated hydroxylamine. Therefore, a probable reactivity towards superoxide radical was to a certain extent, expected. Even if the superoxide scavenging ability of nitroxides **1** and **4** had already been ascertained in a previous study,[31] here their efficacy was compared with nitroxides **2** and **3**. The short chain derivatives, **1** and **4** seem to be better scavengers of superoxide than the longer chain analogues of the indolinic series **2** and **3**. From the chemical point of view, the reactivity of nitroxides **1–4** towards superoxide radical should be similar. In fact, on the basis of the redox potentials for the couple $N\text{–}O^{\bullet}/N\text{–}O^{-}$ indolinic and quinolinic nitroxides are capable of oxidizing superoxide radical to oxygen.[11] Therefore the differences observed between the nitroxides must be ascribed to the fact that **2** and **3** are more hydrophobic and so less soluble and this could limit their interaction and hence reactivity towards superoxide radical generated in the aqueous medium.

Previous studies have demonstrated the antioxidant efficacy of various indolinic and quinolinic nitroxide derivatives in different biological systems. They were seen to inhibit both lipid and protein oxidation of rat liver microsomes and human lipoproteins subjected to oxidative stress,[16,39,40] and to decrease the degree of carbonyl residues formation in oxidised bovine serum albumin.[41] Furthermore, they protected linolenic acid peroxidation by diminishing the amount of TBARS formation and oxygen consumption.[42] Therefore, the results obtained in this study on the hemolysis of trout erythrocytes were surprising, since they were expected to protect against erythrocyte hemolysis as had already been observed on human RBCs.[31] Instead, the presence of nitroxides in trout RBCs suspension promoted hemolysis, i.e. they reduce the half-time ($t_{1/2}$) of the hemolytic process and this effect is more pronounced for compounds **1** and **4** (Table I). The longer chain analogues of nitroxide **1**, also reduce $t_{1/2}$ but to a much lesser

extent. To address the question of why nitroxides **1** and **4** enhanced the degree of trout erythrocyte hemolysis, experiments were undertaken to determine their effect at the membrane level before the hemolytic event, i.e. after 1 h of incubation. Since nitroxides are lipophilic compounds they are expected to localize within the lipid bilayer of the erythrocyte membranes, therefore fluorescence studies and conjugated diene evaluation were carried out on liposomes made of lipids extracted from erythrocyte membranes.

Fluoresence measurements were performed using laurdan as a probe to report changes on membrane polarity and on the physical state of membrane phospholipids before the hemolytic event. The data are expressed as generalized polarization that is correlated with the polarity and physical state of the phospholipid environment surrounding the probe, as reported by Parasassi *et al.*[23,24] In particular, high GP values correspond to a less polar environment and a shift of phospholipids towards a gel phase, while low GP values are correlated with a more polar environment and a shift towards a liquid-crystalline phase, which is the more natural physical state of membrane phospholipids.[23,24] The data reported in Table II show that GP_{340} increases after 1 h of incubation in the control samples in which nitroxides **1** and **4** were excluded while this value is reduced when the nitroxides are present in trout erythrocyte suspensions. This means that the nitroxides present in the erythrocyte suspension do not alter the physical state of the membrane phospholipids incubated in conditions of thermal and oxidative stress. Therefore, the nitroxides protect membrane phospholipids by maintaining them in a liquid-crystalline phase before the hemolytic event.

The relative abilities of nitroxides **1** and **4** to inhibit the early stages of lipid peroxidation of trout erythrocyte membranes before hemolysis was monitored by observing their effects on conjugated diene formation (Table III). An increase in the ratio A_{233}/A_{215} is correlated with a high conjugated diene formation which is an index of the degree of lipid peroxidation. The presence of the nitroxides (100 μM) in the erythrocyte suspension protects membranes from oxidation since the degree of conjugated dienes does not increase compared to the control and this is due to the radical-scavenging/antioxidant ability of indolinic and quinolinic nitroxides.[36–38]

From the results presented above, it would seem that the nitroxides have no perturbating effect on the membranes before hemolysis, since in their presence, lipid peroxidation is inhibited and the physical state of the phospholipids remains unchanged. However, hemolysis is enhanced in the presence of these compounds. Since oxidized hemoglobin i.e. met-Hb, may play an important role in the initiation and development of lipid peroxidation after coming into contact with membrane lipids, experiments were undertaken in order to determine the possible influence that these nitroxides could have on the stability of both, human and trout hemoglobins. As can be observed from the results reported in Table IV, nitroxides **2** and **3** do not influence the hemoglobin oxidation rate. However, both compounds **1** and **4** remarkably increase the autoxidation rate of the three hemoglobins tested in a relatively short time compared to the controls, and this is in accord with a study reported by Grinberg and Samuni in which the piperidine nitroxide TEMPO also facilitated the oxidation of oxy-Hb to met-Hb.[43] Secondly, another possible explanation could be due to the fact that **1** and **4** being smaller sized molecules, could enter the heme pocket easier and therefore promote hemoglobin oxidation to a larger extent with respect to compounds **2** and **3** which are larger molecules due the presence of the longer acyl chain. It is worth noting the high oxidation rate of trout hemoglobins compared to human hemoglobin. This finding could be one of the possible reasons to explain the increased hemolysis of trout erythrocytes in the presence of nitroxides **1** and **4**. Previously, we reported that human RBCs subjected to oxidative stress were protected when the nitroxides were present in the incubation

medium[31] contrary to what has been observed in this study on trout RBCs. However, it must be borne in mind that the results presented here regard trout erythrocytes, nucleated cells, which are different from other RBCs, and this might be a reason for the contrasting results observed. Nevertheless, the experiments carried out using erythrocytes saturated with carbonmonoxide which binds to hemoglobin stabilizing it,[19] and in the presence of nitroxides **1** and **4**, shows that the rate of hemolysis is reduced but not completely to the values of the controls. This implies that hemolysis of trout RBCs does not totally depend on the formation of met-Hb in the presence of nitroxides.[43] This effect was also observed in a previous study where various N-acylethanolamines were used.[44]

At this stage, it is difficult to determine what is the exact molecular mechanism responsible for the early hemolytic event of erythrocytes in the presence of nitroxides **1** and **4**. Future studies will be necessary to address this question. In conclusion, it seems that these nitroxides exert their antioxidant activity during the early stages of oxidative stress as confirmed by the fluorescence study and conjugated diene formation, and that at a later stage, this antioxidant activity becomes overwhelmed by other mechanisms, besides the oxidation of hemoglobin, which trigger a strong oxidative insult. Since the chemical reactivity of these nitroxides is extremely similar, the different behaviour of the studied compounds is likely due to their physical interaction with the system under study.

Acknowledgements

The authors thank the "Italian Ministero dell'-Università e della Ricerca Scientifica e Tecnologica (MURST)" for financial support.

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