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Short Communication

**THE EFFECT OF PEROXYNITRITE AND SOME ANTIOXIDANTS ON
THE RATE OF OSMOTIC HEMOLYSIS OF BOVINE
ERYTHROCYTES**

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Abstract: Bovine erythrocytes treated with peroxynitrite (ONOO⁻), a cytotoxic species formed *in vivo* via the reaction of nitric oxide (NO[•]) and the superoxide anion (O₂^{-•}), show an increased rate of hemolysis on sudden osmotic stress. The increase in the rate was peroxynitrite concentration dependent. In the presence of some antioxidants (uric acid, ascorbic acid, glutathione, melatonin and albumin), this effect was significantly lower, with ascorbic acid as the most efficient antioxidant.

Key Words: Peroxynitrite, Erythrocyte, Hemolysis, Antioxidant, Stopped-flow Technique

INTRODUCTION

Peroxynitrite (ONOO⁻), formed *in vivo* in the reaction of nitric oxide (NO[•]) and the superoxide anion (O₂^{-•}), is thought to be the main agent responsible for nitric oxide toxicity [1]. ONOO⁻ is a stable anion in alkaline solution (pK of 6.8); however, upon protonation, ONOOH decays rapidly, generating reactive species that readily react with various biomolecules [1-3]. The erythrocytes may be the main target for peroxynitrite formed by the endothelial cells of blood vessels. It was found that the action of peroxynitrite on erythrocytes caused oxidation of intracellular glutathione, peroxidation of membrane lipids, aggregation and nitration of membrane proteins, inactivation of acetylcholinesterase [4]. To

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Abbreviations used: 5DSA - 5 doxyl stearic acid; 16DSA - 16 doxyl stearic acid; EPR - electron paramagnetic resonance; UA - uric acid; AA - ascorbic acid; GSH - reduced glutathione; Mel - melatonin; Alb - bovine serum albumin; Pla - blood plasma.

check how the peroxynitrite-related modifications of the erythrocyte membrane influence the dynamic process of membrane rupturing, which takes place during osmotic hemolysis, we investigated the rate of hemolysis of peroxynitrite-treated bovine erythrocytes exposed to a sudden osmotic stress in the presence of some natural antioxidants.

MATERIALS AND METHODS

Uric acid, ascorbic acid, glutathione, melatonin, bovine serum albumin, 5- and 16 doxyl stearic acids (5DSA, 16DSA) were purchased from Sigma Chemical Co. Peroxynitrite was synthesized by azide-ozone reaction [5]. Bovine erythrocytes were isolated from fresh blood (with citrate as the anticoagulant), and washed with 147 mM NaCl/5.6 mM phosphate buffer, pH 7.4. The erythrocytes (1.5% hematocrit) were treated with peroxynitrite in 113.5 mM NaCl/30 mM phosphate buffer, pH 7.4, in the absence/presence of antioxidants. As a control for the potential effects of the products of peroxynitrite decomposition, peroxynitrite was allowed to decompose for 5 min in the phosphate buffer (pH 7.4). The apparent rate of osmotic hemolysis was measured by computer-controlled universal electrochemical meter connected with a stopped-flow device, allowing a rapid mixing of the isotonic cell suspension (hematocrit 0.06%) with an equal volume of water. The apparent rate of hemolysis was defined as the steepest slope of the semilogarithmic plot of the decrease in turbidity (measured at 700 nm) that takes place during hemolysis [6]. The EPR spectra of the spin-labeled (5DSA, 16DSA) erythrocyte ghosts, prepared according to [7] and then treated with peroxynitrite, were recorded on a Bruker ESP-300E X-band spectrometer. The order parameter (S) for 5DSA and the rotational correlation time (τ_R) for 16DSA were calculated [8].

RESULTS AND DISCUSSION

Exposing erythrocytes to peroxynitrite increased the rate of osmotic hemolysis in a concentration-dependent manner (Fig. 1.). This increase was due to the action of peroxynitrite and not due to impurities or decomposition products contained in the peroxynitrite preparation (data not shown). The effect of peroxynitrite on the relative rate of hemolysis (with respect to the appropriate control) was greater after the erythrocytes were incubated at 37°C for 20 min (Fig. 2.). This effect was because of a decrease in the apparent rate of hemolysis that takes place in control cells during incubation (compare boxes P20/P0 and C20/C0 in Fig. 2). Since in the course of hemolysis, the erythrocyte membrane bilayer is ruptured and its stability can be reflected in its fluidity [6], we checked the EPR spectra of spin labels (5DSA and 16DSA) incorporated into the lipid bilayer of the erythrocyte membranes treated with peroxynitrite. The preliminary results show that in peroxynitrite-treated membranes, fluidity was markedly decreased in the hydrophobic core of the bilayer (Fig. 3.).

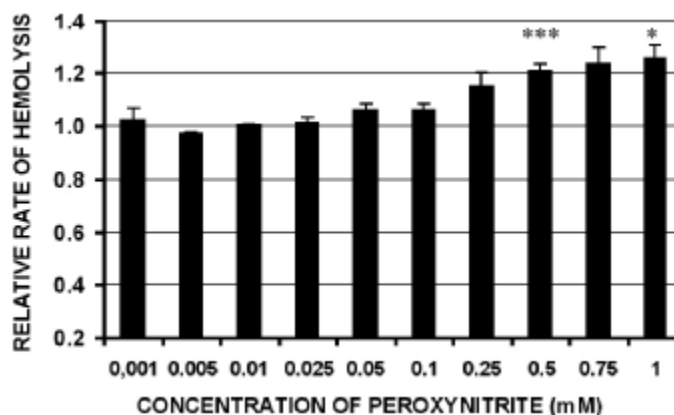


Fig. 1. The effect of peroxy-nitrite concentration on the relative rate of osmotic hemolysis of bovine erythrocytes. The rate of hemolysis was measured immediately after peroxy-nitrite treatment. Relative values, with respect to the control (untreated cells) are the means \pm SE of 2-3 independent experiments (for 0.5 mM – n=19). Statistical significance was determined using Student's t-test (*, $p < 0.05$; ***, $p < 0.001$).

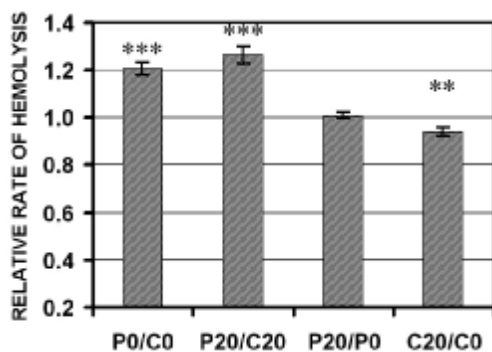


Fig. 2. Relative rates of osmotic hemolysis of bovine erythrocytes treated with 0.5 mM peroxy-nitrite. The rates of hemolysis were measured immediately after peroxy-nitrite treatment and after 20 min incubation at 37°C. P0, P20 – the apparent rate of osmotic hemolysis of erythrocyte treated with peroxy-nitrite measured before and after incubation, respectively. C0, C20 – the apparent rate of hemolysis of control erythrocytes before and after incubation, respectively; C0 = $(0.286 \pm 0.026) \text{ s}^{-1}$. Values are means \pm SE of 14 to 19 independent experiments (**, $p < 0.01$; ***, $p < 0.001$).

Fig. 4. presents the calculated values for the efficiency of antioxidants in protecting erythrocytes from a peroxy-nitrite-induced increase in the rate of hemolysis. The inhibitory effect of antioxidants after 20 min incubation at 37°C is comparable to that observed immediately after peroxy-nitrite addition (data not shown). All the investigated antioxidants decreased peroxy-nitrite effect; ascorbic acid was the most efficient. Because ascorbic acid has a low second order rate

constant for the reaction with peroxynitrite [9], it is unlikely that the inhibition effect is due to a direct reaction with peroxynitrite; it is rather related to reactive oxidizing and nitrating species formed during the reaction of peroxynitrite with biomolecules. The increase in the rate of hemolysis observed in the control erythrocytes suspended in bovine serum albumin is unclear and needs further investigation.

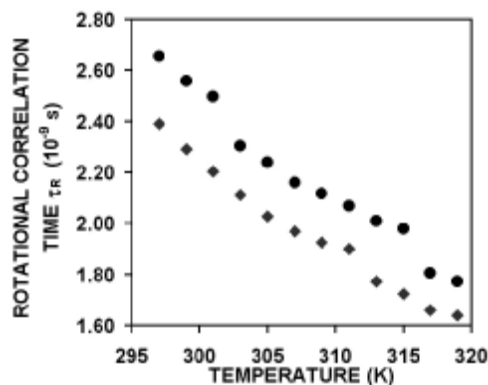


Fig. 3. The effect of temperature on the rotational correlation time τ_R for 16DSA bound to bovine erythrocyte ghosts. (♦) control membranes, (•) membranes treated with 0.5 mM peroxynitrite.

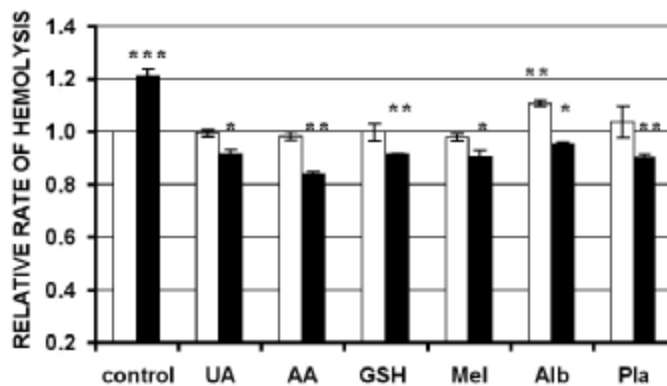


Fig. 4. The effect of antioxidants on the rate of osmotic hemolysis of bovine erythrocytes (control and treated with 0.5 mM peroxynitrite) measured immediately after peroxynitrite treatment. Open boxes: the rate of osmotic hemolysis without peroxynitrite but with antioxidant, with respect to the control (untreated cells). Black boxes: the rate of osmotic hemolysis with peroxynitrite and antioxidant, with respect to peroxynitrite-treated cells. Control, black box: with peroxynitrite with respect to untreated cells. Values are the means \pm SE of 3 to 5 independent experiments, for control $n=19$ (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$). UA, uric acid, 300 μ M; AA, ascorbic acid, 300 μ M; GSH, glutathione, 300 μ M; Mel, melatonin, 300 μ M; Alb, albumin, 5 mg/ml; Pla, blood plasma, 50% (v/v).

In conclusion, although the details of the underlying mechanism are not clear, the presented data show that peroxynitrite can increase the rate of hemolysis of erythrocytes upon sudden osmotic stress, and that this increase is partially suppressed by ascorbic acid, uric acid, glutathione, melatonin, albumin and blood plasma. It is suggested that a peroxynitrite-induced increase in the rate of hemolysis is caused by changes in the physical properties of the membrane (e.g. rigidity of the lipid bilayer and the state of the cytoskeleton). The protective effect of the studied antioxidants might be the result of their different potency to inhibit lipid peroxidation and the aggregation of proteins, in particular spectrin. Since exposure of the erythrocytes to peroxynitrite does not result in any significant spontaneous hemolysis [4], the measurement of the rate of osmotic hemolysis by application of the stopped-flow technique seems to be a convenient method for monitoring the effect of peroxynitrite and various antioxidants on the state of cell membrane, which is directly responsible for the rate at which the cell lysis progress.

Our results also show that membrane modifications occurring immediately after peroxynitrite treatment do not significantly change during further incubation. In many studies concerning the effect of peroxynitrite on erythrocyte membranes, a phosphate buffer with high phosphate concentration is used. The high concentration of phosphate in the incubation buffer, needed to keep pH 7.4 after the addition of the basic peroxynitrite solution, may influence glycolytic activity, osmotic properties, fluidity and skeleton-lipid bilayer interaction in bovine erythrocytes [10]. To avoid false results, the possible effect on the erythrocyte membrane of prolonged incubation with phosphate ions must be always taken into account and monitored using the appropriate controls.

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