ISSN 0006-3509, Biophysics, 2006, Vol. 51, No. 1, pp. 23–28. © Pleiades Publishing, Inc., 2006.
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The Effect of Peroxynitrite on the Affinity of Hemoglobin for Oxygen In Vitro

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Received February 15, 2005

Abstract—The goal of the work was to asses the effect of peroxynitrite on the affinity of hemoglobin for oxygen in in vitro experiments. It was demonstrated that the incubation of whole venous blood with peroxynitrite increased the affinity of hemoglobin for oxygen. Presumably, this effect is realized through generation of various forms of hemoglobin: heme-oxidized and modified at amino acid residues of the protein. The dependence of the results of hemoglobin–peroxynitrite reactions on carbon dioxide tension and the degree of hemoglobin oxygenation is discussed.

Key words: hemoglobin, peroxynitrite, affinity of hemoglobin for oxygen

DOI: 10.1134/S0006350906010040

Peroxynitrite ($ONOO^-$), formed from nitric oxide (NO) and superoxide anion ($O_2^{\bullet -}$), is a highly reactive molecule involved in protective reactions of the body, processes of oxidative stress, apoptosis, and posttranslational modification of proteins [1]. As a strong oxidizing and nitrating agent, peroxynitrite (the joint name for peroxynitrite anion $ONOO^-$ and peroxynitrous acid ONOOH) is capable of reacting with amino acids, such as tyrosine, tryptophan, cysteine, and methionine, as well as with nucleic acids, membrane lipids, and metal-containing proteins [2].

The main target of peroxynitrite in blood is hemoglobin. The interaction between peroxynitrite and oxy- or deoxyhemoglobin results in the formation of methemoglobin [3–5]. Spectroscopy studies have demonstrated that peroxynitrite oxidizes hemoglobin via generation of intermediate hemoprotein forms—ferryl (Fe^{4+}=O) or perferryl ($Por^{\bullet +}$–Fe^{4+}=O) [6]. In addition, the radical $NO_2^{\bullet}$, formed as peroxynitrite breaks down, nitrates the tyrosine residues in hemoglobin [7]. In the presence of physiological concentrations of carbon dioxide (CO_2), $ONOO^-$ generates the nitrosoperoxycarboxylate anion adduct ($ONOOCO_2^-$), which breaks down to oxidize hemoglobin and form the nitrating radical $NO_2^{\bullet}$ [8]. Metal-containing compounds, for example, hemoglobin, are capable of increasing the generation of nitrotyrosine via a bimolecular reaction with peroxynitrite [2]. In addition, the metal of the heme group, which is further reduced, is able to substantially elevate the nitration of tyrosine residues, which allows the metal-containing substances to be regarded as a catalyst of nitration [2]. Each polypeptide chain of hemoglobin contains three tyrosine residues. However, only some of these amino acid residues are the most probable targets for nitration [9]. Taking into account the locations of tyrosine residues in the three- and four-dimensional structures of the protein, their distance from heme, and several other factors, it is hypothesized that nitration is a selective process that leads to certain definite changes in the conformation of the overall hemoglobin molecule [2, 10]. Presumably, hemoglobin is involved in the protection of extra- or intracellular structures against their damage by peroxynitrite [11]. It is also assumed that the modification of hemoglobin by nitric oxide or peroxynitrite may influence certain characteristics of this hemoprotein, in particular, its oxygen-binding properties [12–14]. Consequently, the goal of our experiment was to assess the effect of peroxynitrite on the affinity of hemoglobin for oxygen (AHO) in in vitro experiments.

EXPERIMENTAL

In vitro experiments. The heparinized blood of rabbits weighing 3.5–4.5 kg (n = 33) was used in the experiment. Animals anesthetized with thiopental (50 mg/ml) were bled using catheterization of the jugular vein. Immediately after being taken, the blood was stored under anaerobic conditions at a temperature of +1°C and used for experiments during the first 5–6 h after collection. The suspension of erythrocytes washed with isotonic sodium chloride solution was prepared

Abbreviations: AHO, affinity of hemoglobin for oxygen; ODC, oxyhemoglobin dissociation curve; OTF, oxygen transport function.

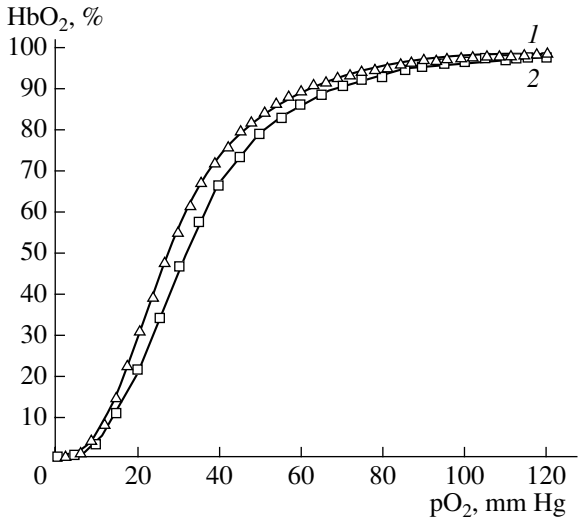

Fig. 1. Position of oxyhemoglobin dissociation curve under standard conditions (pH 7.4, pCO_2 = 40 mm Hg, 37°C) after incubation of the whole venous blood with peroxynitrite for 30 min at 37°C: (*1*) experiment and (*2*) control.

either in 0.15 M sodium phosphate buffer (pH 7.4) or by resuspending erythrocytes in plasma centrifuged twice to remove leukocytes and platelets. The suspensions contained 40% erythrocytes.

In the first set of experiments, the whole blood and the erythrocyte suspension in the buffer or plasma were incubated for 30 min at 37°C under anaerobic conditions with peroxynitrite at a ratio of hemoglobin (tetramer) to $ONOO^-$ of 1 : 1. In the second set of experiments, the samples were incubated with peroxynitrite at a ratio of $ONOO^-$ to hemoglobin (tetramer) of 1 : 2 for 30 min. In the latter set, oxygenation or deoxygenation of the samples for 30 min preceded the incubation of blood or suspension with peroxynitrite. In the third set of experiments, the whole blood supplemented with peroxynitrite was incubated for 10 min in a water bath at 48°C. In another experiment of the same set, the blood was deoxygenated with a mixture of 94.5% N_2 and 5.5 CO_2 for 30 min prior to the incubation at 48°C. In all the experiments, the blood mixed with isotonic 0.9% NaCl solution or the corresponding buffer was used as a control.

Determination of the affinity of hemoglobin to oxygen, blood pH, and blood gas composition. The characteristic used to assess AHO was *p*50, the value of oxygen partial pressure when the degree of hemoglobin oxygenation is 50%. AHO was characterized by two values—the standard p50, measured at pH 7.4, pCO_2 = 40 mm Hg, and t = 37°C, and the actual *p*50 for actual values of pH, pCO_2, and temperature. The *p*50 was determined in an ABL-330 (Radiometer, Denmark) gas microanalyzer. The oxyhemoglobin dissociation curve (ODC) was calculated according to Hill's equation. The tensions of carbon dioxide (pCO_2) and oxygen (pO_2) and blood pH were also measured using ABL-330.

Determination of NO_x. The concentrations of nitrites and nitrates in blood plasma and hemolysate were determined using the Griess reagent [15]. Initially, the proteins of probes were precipitated with 30% $ZnSO_4$. After centrifugation, the supernatant was incubated for 12 h with metallic cadmium to reduce nitrates to nitrites. Then, the Griess reagent was added to determine spectrophotometrically the total concentration of nitrates and nitrites in the sample.

Determination of the methemoglobin concentration in the sample was performed using the Drabkin reagent.

Reagents. *L*-arginine and nitroarginine methyl ester (Sigma, United States) were used in the experiment. Peroxynitrite was obtained by mixing 0.6 M sodium nitrate solution and 0.6 M hydrogen peroxide solution in 0.7 M hydrochloric acid followed by stabilization in 0.9 M sodium hydroxide solution [16]. The concentration of peroxynitrite in solution was assessed spectrophotometrically according to the absorption at a wavelength of 302 nm ($\varepsilon_{302\ nm}$ = 1679 M^{-1} cm^{-1}).

Statistical processing of the results was performed using the Statistica program.

RESULTS

The effects of peroxynitrite on the characteristics of the oxygen transport function (OTF) of the whole blood and erythrocyte suspensions in buffer and plasma. Initially, peroxynitrite was added to the whole blood at a ratio of 1 : 1 with respect to hemoglobin and incubated for 30 min at 37°C. In this sample, a decrease in the standard *p*50 by 3.65 ± 1.28 mm Hg ($p < 0.05$) and the actual *p*50 by 4.47 ± 1.50 mm Hg ($p < 0.05$) was recorded (Fig. 1). The content of methemoglobin in this case increased by 354% ($p < 0.05$) compared with the control. An increase in the nitrite concentration in plasma exceeded the control by 416.52% ($p < 0.05$), although this characteristic remained the same in erythrocytes (Table 1).

Addition of peroxynitrite to the erythrocyte suspension prepared in buffer or plasma caused no changes in OTF. In the suspension prepared in 0.15 M sodium phosphate buffer, the total content of nitrites in erythrocytes increased by 82.98% ($p < 0.05$), and in the buffer, by 579.15% ($p < 0.05$), while the concentration of hemoglobin increased only by 178.65% ($p < 0.05$) (Table 1).

In the erythrocyte suspension in plasma, there was a significantly higher total concentration of nitrites in the erythrocyte mass (by 73.34%, $p < 0.05$), as well as a significantly higher amount of methemoglobin (by 173.55% at $p < 0.05$) (Table 1).

Interestingly, the pCO_2 in all suspensions was significantly lower that in whole blood (Table 1). The pH value also decreased when erythrocytes were suspended in plasma or buffer (Table 1).

Table 1. Effect of peroxynitrite on the characteristics of OTF of the blood and erythrocyte suspensions in buffer and plasma during incubation for 30 min at 37°C ($M \pm m$)

Characteristic	Whole blood (Hb : $ONOO^-$ = 1 : 1)		Erythrocyte suspension in 0.15 M sodium phosphate buffer (Hb : $ONOO^-$ = 1 : 1)		Erythrocyte suspension in plasma (Hb : $ONOO^-$ = 1 : 1)	
	Control	Experiment	Control	Experiment	Control	Experiment
n	7	7	7	7	7	7
$p50_{st}$, mm Hg	31.63 ± 0.73	27.98 ± 0.41*	23.03 ± 0.99	22.99 ± 0.54	2.993 ± 1.17	30.14 ± 1.87*
$p50_{act}$, mm Hg	37.44 ± 0.99	32.97 ± 0.54*	28.67 ± 1.23	29.21 ± 0.97	36.18 ± 1.11	36.81 ± 2.42
pH	7.323 ± 0.009	7.309 ± 0.011	7.272 ± 0.006#	7.255 ± 0.014#	7.260 ± 0.018#	7.252 ± 0.023#
pO_2, mm Hg	44.70 ± 2.07	48.8 ± 2.08	44.23 ± 2.63	43.74 ± 4.65	49.81 ± 2.50	44.33 ± 4.51
pCO_2, mm Hg	53.10 ± 1.67	51.93 ± 1.34	4.26 ± 0.08#	4.60 ± 0.18#	31.57 ± 1.26#	33.16 ± 1.02#
metHb, %	0.50 ± 0.18	2.27 ± 0.35*	0.89 ± 0.27	2.48 ± 0.49*	1.21 ± 0.39	3.31 ± 0.58*
$C(NO_x^-)$, μmol/l	99.55 ± 20.2	514.2 ± 104.7*	21.1 ± 2.96	143.3 ± 31.56*	390.6 ± 190.8	876.0 ± 262.5
$C(NO_x^-)$ erythr., μmol/l	304.4 ± 54.4	410.9 ± 95.6	648.7 ± 90.44	1187.0 ± 148.7*	741.1 ± 96.3	1284.6 ± 186.6*

Notes: * statistically significant changes relative to the control in each set of experiments at $p < 0.05$; # statistically significant changes relative to the control in the experiment with whole blood in each set at $p < 0.05$.
metHb, concentration of methemoglobin; $C(NO_x^-)$, total concentration of nitrates and nitrites in plasma or buffer; and $C(NO_x^-)$ erythr., total concentration of nitrates and nitrites in erythrocytes.

The effect of peroxynitrite on the characteristics of OTF of the whole blood and erythrocyte suspension in buffer in the case of preoxygenation/predeoxygenation. Oxygenation and deoxygenation of the whole blood increased the methemoglobin concentration relative to the control—by 229.17% ($p < 0.05$) and 614.29% ($p < 0.05$), respectively. The AHO and the total content of nitrites in plasma and erythrocytes in this case remained the same (Table 2).

No changes in pO_2 were observed under any conditions of oxygen supply despite the increase in methemoglobin concentration by 825% ($p < 0.05$) and in the total content of nitrites in erythrocytes by 35.58% ($p < 0.05$), observed during deoxygenation. During oxygenation, the content of NO_x^- increased by 68.31% ($p < 0.05$) in buffer and by 156.12% ($p < 0.05$) in erythrocytes of the same sample (Table 2).

The effect of peroxynitrite on the characteristics of OTF of blood incubated at 48°C. The incubation of whole blood for 10 min at 48°C did not cause any changes in the standard AHO characteristic, the total content of nitrites in plasma and erythrocytes, or the content of methemoglobin in samples; however, the pCO_2 in the sample was elevated by 16.51% ($p < 0.05$) (Table 3). Mixing of blood with peroxynitrite at 48°C caused a significant decrease in the standard $p50$ by 2.37 ± 0.90 mm Hg ($p < 0.05$) relative to the control (48°C) (Fig. 2) on the background of an increasing content in methemoglobin, by 1931.25% ($p < 0.05$). There was as well a decrease in the total concentration of nitrites in plasma by 610.22% ($p < 0.05$) and in erythrocytes by 233.36% ($p < 0.05$) (Table 3). In the experiment with peroxynitrite, the value of pCO_2 increased compared with the control at 37°C by 9.86% ($p < 0.05$) (Table 3). Predeoxygenation at 48°C resulted in an increase in the methemoglobin content by 692.86 ($p < 0.05$); NO_x^- in plasma, by 2199.04% ($p < 0.05$); and NO_x^- in erythrocytes, by 513.62% ($p < 0.05$) (Table 3). However, the standard characteristic—the affinity of hemoglobin to oxygen—did not differ from the control.

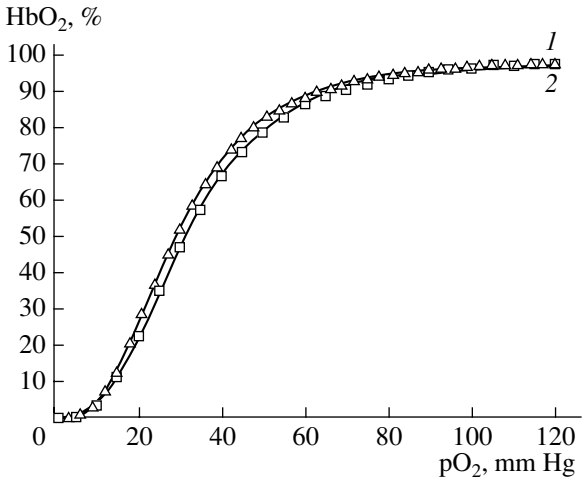

Fig. 2. Position of oxyhemoglobin dissociation curve under standard conditions (pH 7.4, pCO_2 = 40 mm Hg, 37°C) after incubation of the whole venous blood with peroxynitrite for 10 min at 48°C: (*1*) experiment and (*2*) control.

Table 2. Effect of peroxynitrite on the characteristics of oxygen transport function of the blood and erythrocyte suspensions in buffer and plasma in the case of preoxygenation/predeoxygenation during incubation for 30 min at 37°C ($M \pm m$)

Characteristic	Whole blood with preoxygenation (Hb : $ONOO^-$ = 2 : 1)		Whole blood with predeoxygenation (Hb : $ONOO^-$ = 2 : 1)	
	Control	Experiment	Control	Experiment
N	7	7	7	7
$p50_{st}$, mm Hg	30.23 ± 0.22	29.96 ± 0.46	31.70 ± 1.05	30.78 ± 0.96
$p50_{act}$, mm Hg	35.16 ± 0.30	34.99 ± 0.59	37.35 ± 1.00	36.99 ± 1.35
metHb, %	0.24 ± 0.09	0.79 ± 0.10*	0.014 ± 0.013	0.94 ± 0.06*
$C(NO_x^-)$, μmol/l	117.3 ± 41.4	266.0 ± 148.2	145.83 ± 77.77	88.9 ± 15.1
$C(NO_x^-)$ erythr., μmol/l	360.03 ± 67.5	411.57 ± 81.91	348.0 ± 69.8	365.0 ± 55.49
Characteristic	Erythrocyte suspensions with preoxygenation (Hb : $ONOO^-$ = 2 : 1)		Erythrocyte suspensions with predeoxygenation (Hb : $ONOO^-$ = 2 : 1)	
	Control	Experiment	Control	Experiment
N	6	6	6	6
$p50_{st}$, mm Hg	23.44 ± 0.59	23.44 ± 0.96	23.39 ± 0.58	24.40 ± 0.86
$p50_{act}$, mm Hg	25.63 ± 0.60	25.97 ± 1.21	25.63 ± 0.73	26.85 ± 1.00
metHb, %	0.55 ± 0.19	1.92 ± 0.65	0.16 ± 0.07	1.48 ± 0.23*
$C(NO_x^-)$, μmol/l	16.82 ± 1.73	28.31 ± 3.89*	18.28 ± 3.9	26.68 ± 3.64
$C(NO_x^-)$ erythr., μmol/l	150.67 ± 10.52	385.9 ± 49.38*	289.8 ± 29.87	392.9 ± 32.8*

Notes: * statistically significant changes relative to the control in each set at $p < 0.05$.
metHb, concentration of methemoglobin; $C(NO_x^-)$, total concentration of nitrates and nitrites in plasma or buffer; and $C(NO_x^-)$ erythr., total concentration of nitrates and nitrites in erythrocytes.

DISCUSSION

In our experimental model, sufficiently high ratios of $ONOO^-$ to Hb were used, since the concentrations of nitric oxide in the parts of the vascular system, such as arterioles and capillaries, are considerably higher than in the blood of large blood vessels. This is explainable by the existing differences in the NO synthase activity of endothelium throughout the vascular system, which is maximal in the arterioles and considerably decreased in veins, as was confirmed by immunohistological studies [17]. In addition, the ratio of the blood volume of the terminal arterioles and capillaries to the surface area of these vessels is the smallest [12]. This suggests that the concentration of nitric oxide in the microcirculatory bloodstream is higher than in other parts of the vascular system [12]. Similar to other blood cells, the circulating platelets are able to generate sufficiently high concentrations of oxygen free radicals [18, 19]. The interaction at the level of capillaries between the oxygen free radicals and the amounts nitric oxide present there lead to generation of considerable quantities of peroxynitrite.

It is known that the interaction between hemoglobin and peroxynitrite may lead to the oxidation of heme or to the modification of tyrosine and cysteine residues of the protein [5]. Tyrosine nitration is a selective process, which leads to certain definite changes in the protein conformation [9, 20, 21]. It is also assumed that the modification of hemoglobin by peroxynitrite—heme oxidation or modification of the protein part via the nitration of tyrosine—may well influence AHO [12]. The incubation of whole blood with peroxynitrite in our experiments decreased both the standard and actual characteristics of AHO. The leftward shift of OTF correlates linearly with the content of methemoglobin in blood [22]. Presumably, the change in *p*50 under the conditions of our experiment was caused precisely by the presence of the oxidized form of hemoglobin. However, a significant increase in the concentration of ferrihemoglobin in erythrocyte suspension did not change this AHO characteristic. The significance of other NO derivatives of hemoglobin (nitroso- and nitrosylhemoglobin) also cannot be excluded, since it is known that peroxynitrite displays not only a strong oxidative activity, but also is capable of nitrating and *S*-nitrosylating amino acid residues [19]. Presumably, the effect of peroxynitrite on the position of the ODC of whole blood is determined directly by the products of peroxynitrite interaction with hemoglobin.

The main mechanisms of the peroxynitrite reaction with amino acids are realized via the following forms of peroxynitrite: HOONO, $ONOO^-$, and $ONOOCO_2^-$ [2, 3]. Supposedly, the results of these reactions are deter-

Table 3. Effect of peroxynitrite on the characteristics of oxygen transport function of the blood during incubation for 30 min at 37°C ($M \pm m$)

Characteristic	Incubation at t = 37°C	Incubation at t = 48°C	Incubation with peroxynitrite at t = 48°C (Hb : $ONOO^-$ = 1 : 1)	Incubation at t = 48°C with deoxygenation	Incubation with peroxynitrite at t = 48°C with deoxygenation; (Hb : $ONOO^-$ = 1 : 1)
N	8	8	8	8	8
$p50_{st}$, mm Hg	32.13 ± 0.81	31.45 ± 0.52	29.08 ± 0.98#*	30.81 ± 0.49	29.61 ± 0.78*
$p50_{act}$, mm Hg	33.99 ± 1.48	34.19 ± 1.14	31.46 ± 1.36	35.30 ± 1.13	33.63 ± 1.26
pH	7.306 ± 0.026	7.265 ± 0.019	7.282 ± 0.018	–	–
pO_2, mm Hg	30.39 ± 3.39	29.96 ± 4.89	28.43 ± 2.35	–	–
pCO_2, mm Hg	51.53 ± 1.84	60.04 ± 1.68#	56.61 ± 1.06#	–	–
metHb, %	0.50 ± 0.18	0.16 ± 0.12	3.25 ± 0.49#*	0.42 ± 0.16	3.33 ± 0.59#*$
$C(NO_x^-)$, μmol/l	58.63 ± 8.82	52.66 ± 8.0	374.0 ± 49.17*#	44.71 ± 9.35	1027.9 ± 30.2#*$
$C(NO_x^-)$ erythr., μmol/l	244.1 ± 21.4	261.1 ± 20.9	870.4 ± 72.3*#	275.4 ± 26.2	1689.9 ± 183.8#*$

Notes: * statistically significant changes relative to the control in each set at $p < 0.05$; # statistically significant changes relative to the control (t = 37°C) at $p < 0.05$; and $ statistically significant changes relative to the control (t = 48°C) at $p < 0.05$.
metHb, concentration of methemoglobin; $C(NO_x^-)$, total concentration of nitrates and nitrites in plasma or buffer; and $C(NO_x^-)$ erythr., total concentration of nitrates and nitrites in erythrocytes.

mined by factors (pH, pCO_2, and pO_2) that influence the ratio of these peroxynitrite forms [5, 11].

According to the assumption of Exener and Harold [6], hemoglobin in its deoxy form is more efficiently oxidized by peroxynitrite. On the contrary, a decrease in pO_2 under experimental conditions hindered the formation of ferrihemoglobin [11]. Our data on the interaction between hemoglobin and peroxynitrite upon preoxygenation and predeoxygenation of both the whole blood and erythrocyte suspension reflect the leading role of oxygen concentration in hemoglobin oxidation by peroxynitrite.

In analyzing the data, attention should be paid to the role of CO_2. In the presence of physiological concentrations of CO_2, the lifespan of peroxynitrite decreases considerably due to formation of its intermediate with CO_2, the nitrosoperoxycarboxylate anion adduct [23], whose isomerization causes the generation of carbonate anion ($CO_3^{\cdot}$) and $NO_2^{\cdot}$ radical [23]. The $CO_3^{\cdot}$ radical is a more specific oxidant of the aromatic residues than $^{\cdot}OH$ radical; consequently, CO_2 enhances the peroxynitrite-dependent one-electron oxidation of tyrosine residues in proteins [2, 24]. A significant decrease in pCO_2 in erythrocyte suspensions compared with the control sample in the experiment with whole blood is likely to reduce the formation of $CO_3^{\cdot}$ radical, which eventually results in a decrease in nitrotyrosine production. Presumably, it is the nitration of the protein part of hemoglobin potentiated by the generation and further isomerization $ONOOCO_2^-$ that has the highest effect on the position of ODC. It is known that CO_2 is a regulator of hemoglobin oxygen-binding properties. An increase in pCO_2 in the blood causes a decrease in the AHO characteristics. The data obtained when incubating the blood at a temperature of 48°C suggest that AHO decreases only in the presence of peroxynitrite on the background of a fairly high CO_2 concentration, whereas in the control at 48°C, the elevated pCO_2 value relative to the control (37°C) has no effect on the oxygen-binding properties of hemoglobin.

Thus, our results suggest that peroxynitrite is not only a cytotoxic oxidant, but also a factor regulating the oxygen-binding properties of hemoglobin. Evidently, its action is realized via various forms of hemoglobin: heme-oxidized and modified at amino acid residues of protein. Peroxynitrite as a regulator of the affinity of hemoglobin for oxygen goes into action at high concentrations and in the presence of a significant content of carbon dioxide in blood.

ACKNOWLEDGMENTS

The work was supported in part by the Foundation for Basic Research of the Republic of Belarus (project no. BOZ-019).

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