Kinetic regularities of erythrocyte hemolysis and hemoglobin oxidation under the action of sulfur-nitrosyl iron complexes as nitric oxide donors

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The kinetics of erythrocyte hemolysis and intra-erythrocyte hemoglobin oxidation under the action of synthetic sulfur-nitrosyl iron complexes was studied. The complexes capable of releasing nitric oxide due to spontaneous hydrolytic decomposition was studied. The addition of these complexes to a 0.2% suspension of mouse erythrocytes results in hemolysis. The kinetic curves of hemolysis exhibit an induction period, whose duration is different for each complex. The hemolysis is preceded by hemoglobin oxidation with nitric oxide penetrating into the cell. The oxidation of hemoglobin follows the first-order rate equation. The apparent first-order rate constants characterizing the NO-donating ability of each complex were determined. The hemolytic effect of the studied complexes is suggested to be related to the formation of peroxynitrite inside erythrocytes. Peroxynitrite is the cytotoxic product of interaction of nitric oxide and the superoxide radical anion.

Key words: erythrocytes, hemolysis, hemoglobin, sulfur-nitrosyl iron complexes, nitric oxide, peroxynitrite.

Nitric oxide as a signal molecule performs significant biological functions in the organisms. These functions are related, first of all, to the modulation of the blood pressure level, the inhibition of thrombus formation, neurotransmission, and non-specific immune protection.[1] At the same time, an excessive formation of nitric oxide observed in many cases induces cytotoxicity dangerous to human health and acting as a leading pathogenic factor of many inflammatory and neurodegeneratory diseases.[2] As a rule, the main source of cytotoxicity is not nitric oxide *per se* but the products of complicated chemical transformations that occur in the organism due to a high reactivity of NO.[3] Therefore, the study of the chemical and biochemical mechanisms, which account for implementation of the cytotoxic potential of nitric oxide in various types of cells and tissues is especially urgent.

The purpose of the present work is the study of the kinetics and mechanism of erythrocyte hemolysis under the action of the sulfur-nitrosyl iron complexes as nitric oxide donors.

Experimental

Materials. Mice of the line C 57 Bl/6f (age 3 months, weight 18—20 g) were used as the blood source.

The binuclear sulfur-nitrosyl iron complexes (SNIC) with different functional ligands were used as nitric oxide donors.[4] The crystal structure of one of the studied complexes (Pym complex, see below) according to the X-ray diffraction analysis data is shown in Fig. 1. This and all other SNIC are based on the characteristic tetragonal bridged structure including two iron atoms, each of which is bonded to two NO groups, and two sulfur atoms bonded to the functional ligands characteristic of each complex. In this work, we used six such complexes, which incorporated different sulfur-containing ligands: the anionic thiosulfate nitrosyl iron complex (TNIC) $Na_2[Fe_2(S_2O_3)_2(NO)_4]\cdot 4H_2O$, the neutral nitrosyl iron complexes $[Fe_2(SR)_2(NO)_4]$ (R is pyrimidin-2-yl (Pym), 1-methylimidazol-2-yl (MIm), benzothiazol-2-yl (BTz)), and the cationic nitrosyl iron complexes

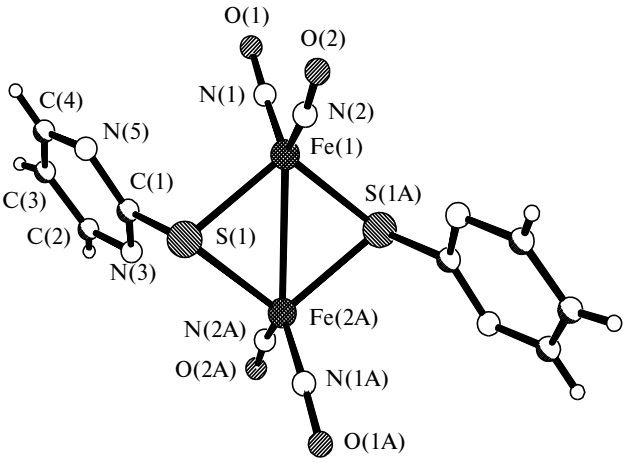

Fig. 1. Crystal structure of $[Fe_2(SR)_2(NO_4)]$, R is pyrimidin-2-yl (Pym).

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$[Fe_2(SR)_2(NO)_4]SO_4 \cdot nH_2O$ (R is cysteinamine, $n = 2$, 5 (Cys) and penicillamine, $n = 5$ (Pen)). No special activation (photo-, thermal, and enzyme activation) is needed to isolate nitric oxide by these complexes: it occurs spontaneously during the hydrolytic decomposition of the complex.[5] The complexes were introduced into an erythrocyte suspension in aqueous solutions (TNIC, Cys, and Pen) or in DMSO (BTz, MIm, and Pym), which were prepared prior to experiment. The weight concentration of DMSO in the sample did not exceed 3%.

Preparation of the erythrocyte mass. Blood was taken from a mouse pre-narcotized with ether using decapitation. A 0.11 *M* solution of sodium citrate was used as an anticoagulant. The blood was collected in a vial to which a solution of the anticoagulant was preliminarily added in the ratio sodium citrate : blood = 1 : 5.

The blood was centrifuged at 1500 *g* at 4 °C for 10 min. The plasma was decanted, and the erythrocyte precipitate was carefully re-suspended in an isotonic solution of NaCl (0.85% NaCl containing a 5 m*M* Na phosphate buffer, pH 7.4). The centifugation procedure was repeated three times. After each centrifugation, the supernatant was decanted, and the erythrocyte precipitate was re-suspended in a new portion of the isotonic solution of NaCl. The erythrocyte mass obtained after the last centrifugation was stored at 4 °C and used in experiments during 36 h.

Hemolysis of erythrocytes. A suspension of erythrocytes, which was prepared prior to experiment by making a 1 : 500 dilution of the erythrocyte mass with the isotonic solution of NaCl, was used. The cell concentration in the obtained suspension was determined by counting in the Gorjaev count chamber. The obtained value was adjusted to the value standard for all experiments: $4.4 \cdot 10^7$ cells in 1 mL (approximately corresponds to haematocrite $H = 0.2\%$) by additional dilution. Hemolytic experiments were carried out in a thermostat at 37 °C with continuous weak stirring. The course of erythrocyte hemolysis was monitored by a change in the absorbance of the suspension at a wavelength of 700 nm. Since this wavelength is beyond the region of electronic absorption of the main molecular component of the system (hemoglobin), the light flow is weakened almost completely due to light scattering on erythrocytes. The absorbance of an erythrocyte suspension at $\lambda = 700$ nm for the dilution level used depends linearly on the fraction of destroyed cells.[6,7] The degree of hemolysis (γ) of an erythrocyte suspension was determined from the ratio

$$\gamma = \frac{A_0 - A}{A_0 - A_{H_2O}}, \qquad (1)$$

where A_0 and A are the absorbances of the reference and experimental SNIC-containing sample, respectively; and A_{H2O} is the absorbance of the sample under the conditions of complete lysis of all erythrocytes with distilled water. In all experiments the absorbance of the reference sample (A_0) was 0.8.

Determination of methemoglobin. Aliquots of 0.4 mL were taken from an erythrocyte suspension ($3.5 \cdot 10^8$ cell mL^{-1}), which was carefully stirred magnetically at 37 °C. Distilled water (1.4 mL) was added to each aliquot, the solutions were allowed to stand for 1 min until hemolysis completed, and the absorbance at 630 nm was measured. The methemoglobin concentration was determined by using the following equation:

$$[HBFe^{3+}] = \frac{\Delta A_{630}}{\varepsilon_{met} - \varepsilon_{oxy}} d, \qquad (2)$$

where ΔA_{630} is the increase in the absorbance of the sample at 630 nm; $\varepsilon_{met} = 3.8$ mmol^{-1} cm^{-1} and $\varepsilon_{oxy} = 0.11$ mmol^{-1} cm^{-1} are the molar absorption coefficients[8] of methemoglobin and oxyhemoglobin at 630 nm, respectively; and d is the dilution factor in the cell.

Results and Discussion

The influence of the SNIC on erythrocyte hemolysis was studied over a wide concentration range. Five of six studied complexes showed a hemolytic effect, an extent of which depends on the concentration of the complex. The kinetic curves of erythrocyte hemolysis obtained at the same concentration of the studied compounds ($4 \cdot 10^{-5}$ mol L^{-1}) are presented in Fig. 2. The duration of the induction period of hemolysis (I) defined as the time of achievement of 10% hemolysis ($\gamma = 0.1$), was used as an individual kinetic characteristic of hemolytic activity of the studied complexes. The induction periods of hemolysis for the corresponding complexes are listed in Table 1. As can be seen from Fig. 2, one of the studied complexes, namely, Pen, showed no hemolytic effect at either the above indicated concentration or at higher concentrations up to 10^{-4} mol L^{-1}.

During the hemolytic experiments, we often observed a change in the color of the erythrocyte suspension occurred before the beginning of hemolysis, which could indicate chemical changes inside the cell. It is known that nitric oxide can rapidly react with oxyhemoglobin in a free solution to form methemoglobin and the nitrate anion.[9] Taking into account that cell membranes are not a serious obstacle for the diffusion of nitric oxide,[10] we could expect that this reaction would occur inside erythrocyte before their decomposition. The spectrophotometric study of the hemolyzates revealed a series of spectral changes indicating the oxidation of oxyhemoglobin to methemoglobin.

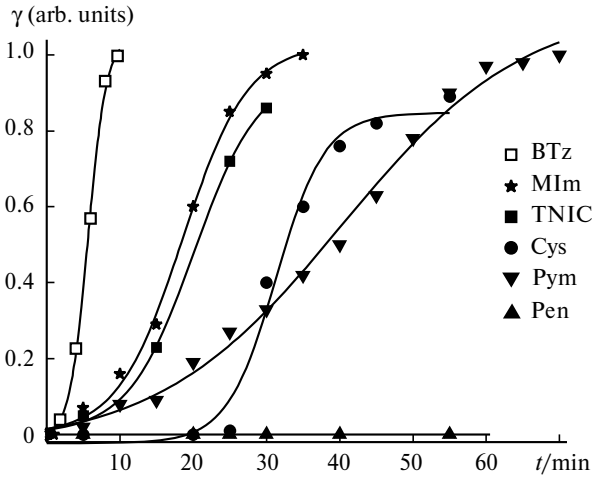

Fig. 2. Kinetics of erythrocyte hemolysis under the action of the SNIC with different functional ligands (erythrocyte concentration in the suspension 0.2%, cell content $4.4 \cdot 10^7$ cell mL^{-1}, 37 °C).

Table 1. Hemolytic activity and NO-donating ability of the SNIC

Complex	Induction period of hemolysis (I/min)	$k \cdot 10^{-3}$ /s^{-1}
Cys	25	1.2±0.8
TNIC	10.5	7.0±1.5
Pym	14	9.6±1.7
MIm	9	11±2.5
BTz	3	8.6±3.5
Pen	—*	55±9

* No hemolytic effect.

The kinetic curves characterizing methemoglobin accumulation inside erythrocytes are shown in Fig. 3. In the presence of each studied compound, methemoglobin is accumulated with different rates, which can be due to the differences in hydrolysis rates of the studied complexes (see Table 3). An analysis of the obtained data showed that the kinetics of methemoglobin formation is well described by the first-order rate equation

$$[HbFe^{3+}] = [HbFe^{3+}]_{\infty} \cdot (1 - e^{-kt}), \quad (3)$$

where t is time, $[HbFe^{3+}]$ is the methemoglobin concentration, k is the first-order reaction rate constant, and $[HbFe^{3+}]_{\infty}$ is the limiting value of methemoglobin concentration at $t \to \infty$.

The first-order rate constants of methemoglobin formation (k) are given in Table 1.

The spontaneous decomposition of the sulfur iron complexes of this class with NO donating[5] obeys the first-order rate equation and the rate constant is close to 10^{-3} s^{-1}.

$$[Fe(SR)_2(NO)_4] \xrightarrow{k_1} \longrightarrow NO + [\text{decomposition products of complex}]. \quad (4)$$

When releasing nitric oxide penetrates into an erythrocyte, it is capable, as already stated above, of reacting with oxyhemoglobin

$$[HbFe^{2+}]O_2 + NO \xrightarrow{k_2} HbFe^{3+} + NO_3^-. \quad (5)$$

When nitric oxide enters into an erythrocyte suspension, this reaction has the rate constant lower than that in the free solution[10] but still considerable and equal to $5.16 \cdot 10^4$ s^{-1}.

The decomposition of the SNIC and the subsequent interaction of the released nitric oxide with hemoglobin inside an erythrocyte can be considered thus as two successive processes characterized by markedly different rate constants: k_2 is seven orders of magnitude higher than k_1. Probably, in this system oxyhemoglobin can act as an efficient trap for nitric oxide released from the SNIC. The rate constants of methemoglobin formation in erythrocytes determined in the present work (see Table 1) can serve as a measure of the NO-donating ability of the studied SNIC of various structures. This characteristic can be used for analysis of a structure—activity relationship in the series of the synthesized complexes and for the preliminary screening of the SNIC for the subsequent comprehensive study of their pharmacological activity.

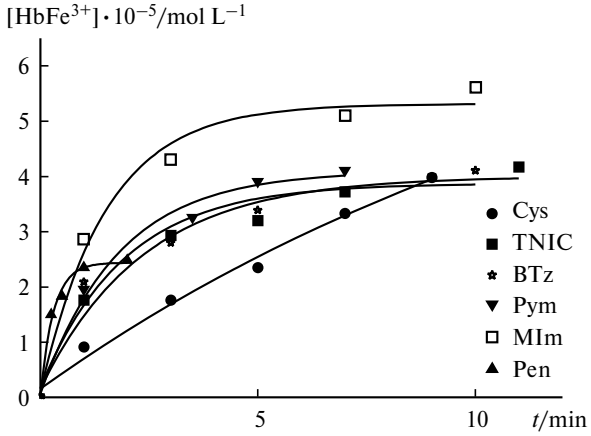

Fig. 3. Kinetics of methemoglobin formation inside erythrocytes under the action of the SNIC (hemoglobin content in the suspension $2 \cdot 10^{-4}$ mol L^{-1}; Pen concentration $2 \cdot 10^{-5}$ mol L^{-1}, concentration of other SNIC $1.2 \cdot 10^{-4}$ mol L^{-1}).

Let us consider the relationship between the NO-donating and hemolytic activities of the studied complexes. As can be seen from Table 1, the weakest NO-donor, Cys ($k_2 = 1.2 \pm 0.8 \cdot 10^{-3}$ s^{-1}), is simultaneously the weakest hemolytic effector (I = 25 min). Four other SNIC, *viz.*, TNIC, Pym, MIm, and BTz, which are stronger NO-donors (k_2 is within $(7—11) \cdot 10^{-3}$ s^{-1}), are stronger hemolytic agents (I ranges from 3 to 14 min). At the same time, the strongest NO-donor Pen ($k_2 = 55 \pm 9 \cdot 10^{-3}$ s^{-1}) showed no hemolytic effect. Thus, the dependence between the rate constants of the NO donating and the hemolytic effect of the studied complexes is described by the curve with a maximum.

There is massive evidence in literature that the main source of cytotoxicity of nitric oxide is the product of the superfast diffusionally controlled bimolecular interaction of nitric oxide with the superoxide radical, *viz.*, peroxynitrite,[11] rather than NO itself

$$NO + O_2^- \longrightarrow HONOO \ (k \sim 10^{10} \text{ L mol}^{-1} \text{ s}^{-1}). \quad (6)$$

The source of superoxide for reaction (6) can be the autooxidation of oxyhemoglobin that continuously occurs in erythrocytes

$$[HbFe^{2+}]O_2 \longrightarrow HbFe^{3+} + O_2^-. \quad (7)$$

Taking into account the published data on the hemolytic effect of synthetic peroxynitrite in the *in vitro* system,[12] we may assume that peroxynitrite formed in reaction (6) can act as a direct initiator of hemolysis. The rate

of peroxynitrite formation in the cell depends, evidently, on the ratio of reactions (5), (6), and (7). In particular, reactions (5) and (7), in which oxyhemoglobin is a common initial reactant, can compete with each other. In this case, the consumption of oxyhemoglobin in reaction (5) can decrease the possibilities of the cell to form the superoxide radical anion in reaction (7), which should result, in turn, in a decrease in the level of peroxynitrite formation by the cell in reaction (6). Taking this into account, we can explain the curve with a maxima describing the dependence of the NO-donating ability on the hemolytic activity of the studied SNIC. In particular, a reason for the entire absence of the hemolytic effect of the most efficient donor of nitric oxide, Pen, can be the fast consumption of oxyhemoglobin, which is the source of superoxide in erythrocyte.

The results of this work show that the triggering mechanism of erythrocyte hemolysis in the presence of nitric oxide donors is the formation of peroxynitrite in the cell. The results obtained agree with the known concepts about peroxynitrite as an important source of cytotoxicity related to nitric oxide metabolism in living organisms.[13] The cytotoxic potential of peroxynitrite is performed, most likely, through the activation of the oxidative process inside erythrocyte, whose specific mechanism requires further studies.

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