CONTRIBUTION OF THE GASOTRANSMITTER NITRIC OXIDE TO THE STRUCTURAL AND FUNCTIONAL ORGANIZATION OF ERYTHROCYTES UNDER CONDITIONS OF HYPOXIA/REOXYGENATION

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Hypoxia is accompanied by changes in metabolism and cell functioning. Erythrocyte hemoglobin can be involved in adaptation to hypoxia by acting as an oxygen sensor, providing a link between oxygen content and blood circulation. The mechanisms providing this function have not been completely established. The purpose of this study was to evaluate the effect of the gasotransmitter nitric oxide on the structural and functional organization of erythrocytes under conditions of hypoxia/reoxygenation. NO participated in adaptive reactions under hypoxia/reoxygenation conditions by changing hemoglobin conformation, followed by changes in hemoprotein spectral characteristics and hemoglobin affinity to oxygen together with increasing anisocytosis, volume and cell surface. The increase in intracellular NO concentrations under hypoxic conditions was provided by extracellular fluid nitrites. Molsidomine (a NO donor) induced a higher NO increase without involvement of the nitrite reductase mechanism, it caused an increase in the average erythrocyte volume, anisocytosis, and an increase in the cell surface.

Key words: erythrocytes; hypoxia/reoxygenation; nitric oxide; flow cytometry; spectroscopy; molsidomine

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INTRODUCTION

The maintenance of oxygen homeostasis is a necessary condition for the functioning of most cells, the work of their enzymes, the presence of membrane asymmetry, synthetic processes, etc. Under conditions of physiological rest, as well as in the absence of pathology, mitochondria consume more than 90% of the available oxygen to produce ATP through oxidative phosphorylation [1]. The limitation or lack of oxygen is accompanied by changes in metabolism and cell functioning. Impaired oxygen absorption during biological oxidation and/or insufficient oxygen supply to body tissues [2–4], results in hypoxia. At the same time, erythron cells (mature erythrocytes and their precursors — reticulocytes) participate in the systemic mechanisms of regulation of blood affinity for oxygen in mammals, and an increase in NO concentrations during hypoxia and an increase in the p50 value (50% oxygen saturation) [5–7] can be considered as an adaptive response.

Hemoglobin (Hb) of erythrocytes can participate in adaptation to hypoxia, acting as an oxygen sensor [8] and providing a relationship between oxygen content and blood circulation. The mechanisms providing this function have not been fully understood. It is suggested that these include ATP release, S-nitrosohemoglobin (SNOHb) formation [9], and deoxyhemoglobin nitrite reductase activity [10].

Molsidomine a compound with a molecular mass of 242.23 Da ($C_9H_{14}N_4O_4$), is a derivative of sydnonimines, which is used in the treatment of cardiovascular diseases. The therapeutic effect of molsidomine is associated with NO formation from its active metabolite SIN-1 (CAS 16142-27-1). NO, by activating NO-dependent guanylate cyclase, promotes an increase in cGMP, which leads to relaxation of myocytes of the vascular wall. In addition to NO, SIN-1 forms peroxynitrite ($ONOO^-$), which inhibits platelet aggregation [11], affects water-ion homeostasis of cells, causing a concentration-dependent change in both the passive flow of ions through the plasma membrane with the participation of ion exchangers and channels, and water flow through aquaporins and membrane defects; peroxynitrite changes the basic physicochemical parameters of blood cells (the concentration of protons and ATP molecules in the cytosol, the value of the transmembrane potential) and initiates various mechanisms of cell death [12].

It has been found that the formation of the active metabolite of molsidomine occurs predominantly in the liver; this is confirmed by the fact that the concentration of molsidomine remains unchanged and the absence of pharmacological effects in hepatectomized rats [9]. It has been shown that the use of molsidomine in a rat erythrocyte suspension model leads to the formation of NO [13].

Incubation of an erythrocyte suspension in the presence of nitroglycerin does not lead to an increase in reactive nitrogen species, but increases the concentration of methemoglobin (MetHb) and induces lipid peroxidation [14].

In this regard, the goal of the work was to assess the effect of NO on the structural and functional organization of erythrocytes under conditions of hypoxia/reoxygenation.

MATERIALS AND METHODS

The studies were carried out using a suspension of erythrocytes from male volunteers aged 41-47 years (n=22). Venous blood was collected into evacuated tubes using EDTA K_2 as an anticoagulant. The suspension of erythrocytes (a hematocrit of 5%) was obtained after centrifuging blood samples at 400 *g* for 10 min and adding a calcium-buffered solution (pH 7.2), lacking basal fluorescence. The erythrocyte suspension was exposed to hypoxia (30 min) (group 1) or hypoxia (30 min)/reoxygenation for 60 min (group 2).

To assess the mechanisms of the effect of hypoxia/reoxygenation during molsidomine administration, *in vitro* experiments were performed under the following conditions: 5% CO_2 and 4% O_2 (hypoxia) and 5% CO_2 and 14% O_2 (reoxygenation), 37°C. These conditions were created in a glove box.

To assess activity of the L-arginine-NO system of erythrocytes, the NO donor, 2 mM molsidomine (IPOCHEM, Poland), was added to the cell suspension during hypoxia/reoxygenation modeling (groups 3 and 4). NO concentration in cells was determined using 4-amino-5-methylamino-2′,7′-difluorofluorescein (DAF-FM DA). This fluorescent probe penetrates the cytoplasmic membrane and is metabolized by esterases. The product of this reaction then reacts with NO with formation of diaminofluorescein triazole (DAF-2T), which remains in the cell. After excitation by a 488 nm laser the DAF-2T emits in the wavelength range 500-520 nm. Control samples did not contain molsidomine and DAF-FM DA.

The assessment of the spectra of the erythrocyte suspension and the calculation of the hemoglobin fractions of the erythrocyte suspension and hemoglobin solution were carried out according to the formulas [15] with specifications:

$$[HbO_2] = 29.8\times A577 - 9.8\times A630 - 22.2\times A560 \quad (1),$$

$$[HHb] = -1.6\times A577 + 2.5\times A630 - 0.33\times A560 \quad (2),$$

$$[MetHb] = 0.2\times A577 - 0.4\times A630 + 0.33\times A560 \quad (3),$$

where, $[HbO_2]$, [HHb] and [MetHb] are the concentrations of oxy-, deoxy- and methemoglobin, respectively; A577, A630 and A560 are the absorption values at the corresponding wavelengths.

The affinity of hemoglobin for oxygen was determined by the p50 parameter (50% degree of saturation with oxygen) at temperature, pH, pCO_2, and pO_2 of the blood corresponding to the actual experimental conditions of these parameters and was determined by the Severinghaus formula [15].

The fluorescence intensity of DAF-FM DA and simultaneous registration of the absorption spectra of the erythrocyte suspension were carried out on a Biotek Synergy H1 plate spectrofluorometer (BioTek Instruments, USA). In parallel with spectrofluorimetry, intracellular NO was assessed using a FACS ARIA cytofluorimeter (BD Biosciences, USA). The intraerythrocyte fluorescence intensity of DAF-FM DA was determined in normocytes (a subpopulation of erythrocytes carrying glycophorin A on the surface and possessing linear dimensions corresponding to erythrocytes) in each cell [16]. For logical gating of normocytes, monoclonal antibodies to CD 235a were used. At least 40,000 cells were analyzed in each sample.

Optical-morphometric analysis of methanol-fixed and eosin-methylene blue-stained blood preparations was carried out using an Olympus BX-53 microscope (Olympus Corporation, Japan), supplemented with a monochromatic filter with a wavelength of 540 nm. From each preparation, archives of grayscale (8-bit) images of at least 300 red blood cells were created from different (random) sections of the blood smear containing a cell monolayer. The surface area of erythrocytes was evaluated using algorithms of the Diamorph-CITO software (Diamorph, Russia).

Hematological parameters (average erythrocyte volume and the width of distribution of erythrocytes by volume) were determined on a Sysmex 2000i hematology analyzer (Sysmex Corporation, Japan).

Nitrites were evaluated using the Measure-iT™ High-Sensitivity Nitrite Assay Kit (Molecular Probes, USA). Quantitative analysis was carried out after calibration by titration with 11 mM sodium nitrite on a Biotek Synergy H1 plate spectrofluorimeter.

The fluorescence of the kit was assessed with excitation at 365 nm and emission at 450 nm.

The data obtained were processed by methods of variation statistics using the Statistica 10.0 program. Results are presented as median (Me) and 25th and 75th percentiles.

All parameters were checked for normality of distribution using the Shapiro-Wilk test. The significance of analysis of variance for multiple comparisons was assessed using the Mann-Whitney test. Correlation analysis was performed using the Spearman's correlation coefficient.

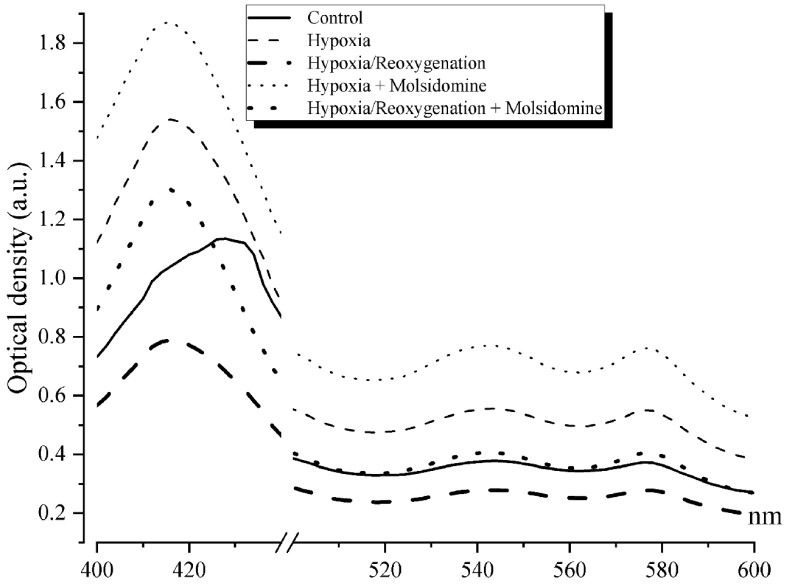

Figure 1. Absorption spectra of blood erythrocytes during hypoxia/reoxygenation and molsidomine application.

RESULTS AND DISCUSSION

Figure 1 shows results of spectrophotometric studies of a suspension of erythrocytes from healthy volunteers during hypoxia/reoxygenation. All the spectra in Figure 1 have characteristic peaks at 540 nm and 576 nm, corresponding to oxyhemoglobin, and a minimum at 560 nm specific for deoxyhemoglobin.

Analysis of the absorption spectra shows that during hypoxia (4% O_2), the oxyhemoglobin fraction (HbO_2) decreases by 9.3% in the control group; by 8.1% and 7.2% in groups 1 and 2, respectively (p<0.05). The proportion of deoxyhemoglobin (HHb) at 4% O_2 in the box atmosphere increased in all groups (p<0.05); There were no differences between the groups. The methemoglobin fraction (MetHb) both in the control and in the main observation groups decreased by 1.0-1.5% (p<0.05).

The reoxygenation process in the control group was accompanied by an increase in the oxyhemoglobin fraction by 26.5%, reaching 51.6% (46.2; 58.4) (p<0.05). There was also a decrease in the deoxyhemoglobin fraction by 9.0% (p<0.05) and an increase in MetHb by 4.7% (p<0.05).

In the main experimental groups, reoxygenation led to an increase in the HbO_2 fraction. Its increase was 5.1% (p=0.07) and 12.5% in groups 1 and 2 (p<0.05), respectively. In contrast to the control the MetHb fraction in the main groups during reoxygenation did not increase, but did decrease. For example, modeling hypoxia/reoxygenation was accompanied by the decrease in the MetHb fraction by 2.4%, and in the case of molsidomine addition by 1.8% (p<0.05). A leftward shift of the absorption maximum of the Soret band was registered in all main observation groups during hypoxia/reoxygenation modeling.

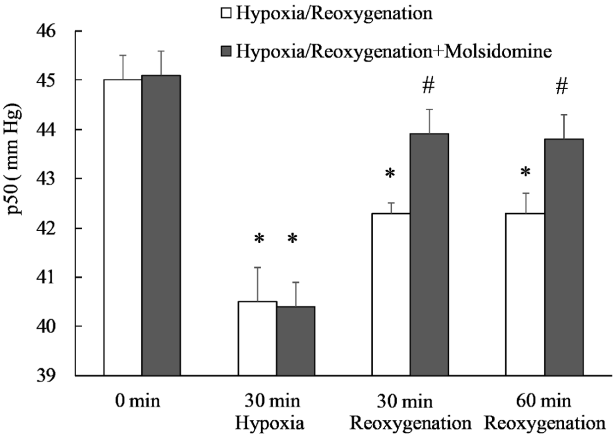

Figure 2. The p50 value during hypoxia/reoxygenation and molsidomine application (Me and 75th percentile). * – p<0.05 as compared to the initial level; # – p<0.05 as compared with hypoxia/reoxygenation.

In the erythrocyte suspension of the control group, O_2 pressure at 50% blood desaturation (p50), was 45.1 (41.8; 36.4) mm Hg. Under conditions of 30-min hypoxia (5% CO_2 and 4% O_2), a decrease in p50 to 40.5 (39.3; 41.7) mm Hg was found in group 1 (p<0.05) and up to 40.4 (39.2; 41.6) mm Hg (p<0.05) when molsidomine was used (group 3). In other words, in the case of hypoxia modeling, the additional use of an NO donor did not affect the affinity of hemoglobin for oxygen (Fig. 2).

Reoxygenation for 30 min was accompanied by a decrease in the affinity of hemoglobin for oxygen: the p50 parameter increased to 42.3 (41.0; 43.6) mm Hg in group 2 and up to 43.9 (42.6; 45.2) mm Hg in the case of molsidomine application (group 3; p<0.05). The same p50 values were maintained during 60-minute reoxygenation (Fig. 2). At the same time, significant differences in the p50 value between the initial state and the hypoxia/reoxygenation

modeling were detected only in group 3. The use of the NO donor during reoxygenation led to a decrease in the affinity of hemoglobin for oxygen as compared to group 3 (p<0.05).

The erythrocyte NO concentration, measured by the fluorescence intensity of DAF-FM DA (RFU_DAF), in the case of hypoxia modeling in the first group increased by 15.7% (p<0.05) (Fig. 3); the dynamics of growth during reoxygenation was preserved and at 60 min, the increase in the erythrocyte NO concentration was 106.4% (p<0.05).

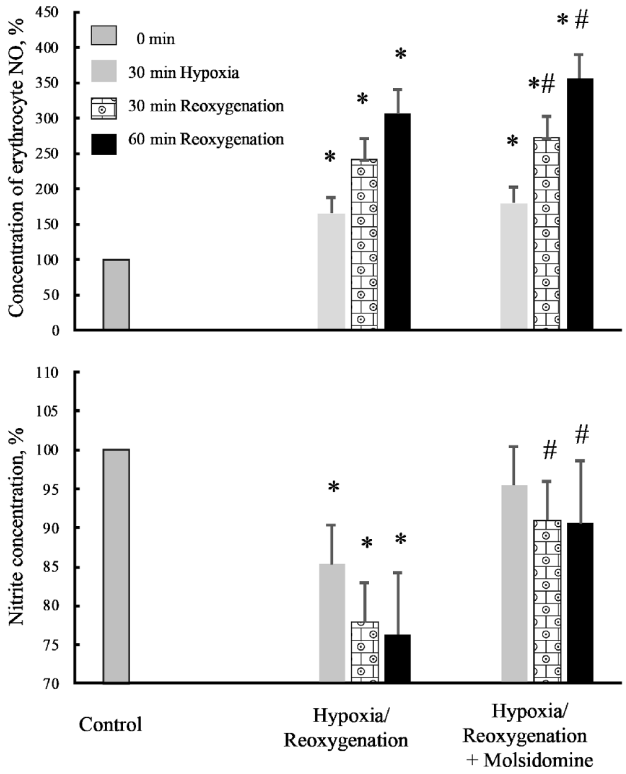

Figure 3. Dynamics of changes in the NO concentration in erythrocytes and nitrites in the extracellular fluid during hypoxia/reoxygenation and molsidomine application (Me and 75th percentile). * – p<0.05 as compared to the initial level; # – p<0.05 as compared with hypoxia/reoxygenation.

The use of molsidomine was accompanied by a higher increase in the NO concentration. For example, during hypoxia (group 3), the increase in NO was 29.1% (p<0.05), and during reoxygenation (group 4) it was 155.5% (p<0.05).

Analysis of nitrite concentrations in samples has shown that hypoxia/reoxygenation modeling leads to a decrease in their concentration (Fig. 3). For example, during hypoxia, the concentration of nitrites in the extracellular fluid decreased by 15.3% (p<0.05), and during reoxygenation by 22.5% (p<0.05).

During hypoxia/reoxygenation under conditions of molsidomine application, a decrease in the nitrite concentration was not detected; statistically significant differences were found only between groups 2 and 4 at the reoxygenation stage (p<0.05). This suggests that the process of erythrocyte adaptation to hypoxia consists in an increase in the intraerythrocyte level of NO, and the main mechanism of its increase includes nitrite reductase reactions involving plasma nitrites. In the presence of exogenous sources of NO, it is probably included in the mechanisms of the erythrocyte adaptation to hypoxia without involvement of the nitrite reductase mechanism, since nitrite consumption is not detected.

A correlation analysis has shown that there is a negative correlation between the concentration of NO in erythrocytes and nitrites in the extracellular fluid (r=-0.71, p<0.05).

According to our data, the NO concentration in the subpopulation of erythrocytes (normocytes) increases during hypoxia/reoxygenation modeling; molsidomine has no additional effect on the increase of erythrocyte NO (Fig. 4). It can be assumed that there are intraerythrocyte homeostatic mechanisms to maintain concentrations of NO and its derivatives under normal and hypoxic conditions.

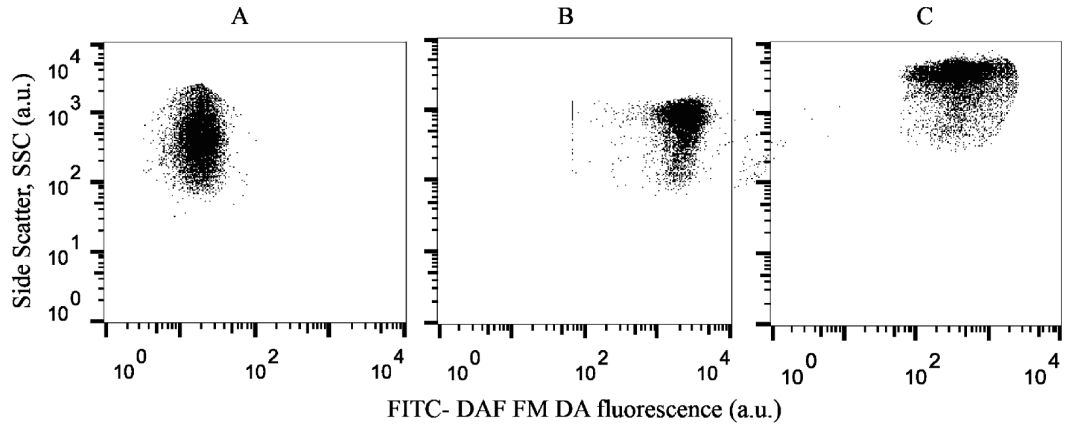

Figure 4. The scattergram of diffractometric parameters and NO content of erythrocytes during hypoxia/reoxygenation and application of molsidomine. **A** (Control) – Median SSC = 323.4 (308.2; 339.1); Median DAF-FM DA = 134.2 (120.2, 147.4); **B** (Hypoxia/reoxygenation) – Median SSC = 400.2 (368.9; 531.1); Median DAF-FM DA = 512.6 (460.8; 563.2)*; **C** (Hypoxia/reoxygenation + molsidomine) – Median SSC = 687.7 (641.3; 719.5)*,#; Median DAF-FM DA = 491.0 (431.9; 550.8)*. * – p<0.05 as compared to the initial level; # – p<0.05 as compared with hypoxia/reoxygenation.

Evaluation of the structural and functional parameters of erythrocytes shows that the addition of molsidomine leads to an increase in side light scattering (SSC) of normocytes (Y axis on the graph), which exceeds both the control values and the value recorded in group 2. In group 4 there was high heterogeneity of the SSC value. At the same time, no increase in the erythrocyte heterogeneity by intracellular NO concentration (X axis) was detected. Adaptive reactions of erythrocytes during hypoxia/reoxygenation modeling include changes in the size and densitometric properties of erythrocytes (Table 1). It has been found that the use of the NO donor led to an increase in the average volume of erythrocytes in combination with an increase in anisocytosis and an increase in the cell surface area.

In the group without the use of molsidomine under conditions of hypoxia/reoxygenation (group 2), there was a decrease in the surface area of erythrocytes and the volume and an increase in the homogeneity of the cell population, both as compared to group 4 and the control.

A number of studies have been devoted to the study of adaptive reactions to hypoxia. The most common systemic response to hypoxia is vasodilation, which provides blood flow and oxygen delivery to tissues using a feedback mechanism in which the target parameters are O_2 or pH [6]. In mammals, vasodilation occurs when hemoglobin saturation decreases from 60% to 40% at a partial pressure of oxygen in the range of 40-20 mm Hg [7].

There is evidence in the literature that erythrocytes participate in the systemic mechanisms of regulation of blood affinity for oxygen [17, 18]. In this case, hemoglobin is an oxygen sensor, and its effector function consists in the allosteric structural transition of the oxygenated (R-state) conformation of hemoglobin to the deoxygenated (T-state) [19]. Good evidence exists for NO participation in systemic adaptation to hypoxia [2–4, 8–10, 20–23]. For example, hemoglobin deoxygenation leads to NO release from the erythrocyte and subsequent NO-dependent vasodilation, but the proposed mechanisms are fundamentally different.

According to the first proposed mechanism, S-nitrosylated hemoglobin (SNO-Hb) releases S-nitrosothiols during deoxygenation, followed by NO formation and vasodilation [2, 3, 11, 24, 25]. Hemoglobin is an allosterically regulated nitrite reductase; it reduces nitrite to NO using the deoxy form of Hb during its deoxygenation [4] thus representing the second possible mechanism of response to hypoxia. Our study showed [16] that when 7% O_2 was used in the model mixture, there was a uniform 4-fold increase in the erythrocyte NO concentration during the whole period of observation. The minimum (less than 2%) oxygen concentration in the experimental gas mixture also led to an increase in NO, but the concentration increase was lower than under other experimental conditions. Reoxygenation for 30 min was accompanied by a sharp increase in the NO concentration, and a 30-min incubation after acute hypoxia led to a higher increase in NO than a 90-min exposure of blood to 7% oxygen.

As follows from the above data, an increase in the NO concentration during hypoxia can be considered as an adaptive reaction, in which the increase in NO and its derivatives in erythrocytes under these conditions can change the dissociation curve of oxyhemoglobin, reducing the affinity of hemoglobin for oxygen [19]. This is consistent with the increase in tissue pO_2 after the use of nitroglycerin in an *in vivo* model [20]. In addition, hypoxia, triggering cellular stress mechanisms, activates nitric oxide synthase (NOS) of erythrocytes; this leads to the release of NO and vasodilation of blood vessels. Taken together, this confirms the important role of the erythrocyte L-arginine-NO-system in the regulation of local blood flow under hypoxic conditions.

An increase in the intracellular NO concentration during hypoxia modeling *in vitro* is accompanied by a decrease in nitrites in the extracellular fluid. At the same time, the use of molsidomine under conditions of increased erythrocyte NO does not lead to changes in nitrite concentrations. A possible explanation for this phenomenon is exogenous NO formed when molsidomine is added; in other words NO is necessary for the adaptation of erythrocytes

Table 1. Optico-morphometric characteristics of erythrocytes during hypoxia/reoxygenation and molsidomine application

Group	Control	Hypoxia/reoxygenation	Hypoxia/reoxygenation + Molsidomine
Area surface, μm^2	141.1 [126.9; 155.1]	120.0 [108.1; 132.1]*	151,2 [135.9; 166.1]$^{\#}$
Mean erythrocyte volume, fl	92.1 [82.9; 101.3]	84.1 [75.6; 92.4]*	113.1 [101.7; 124.3]*,$^{\#}$
Volume erythrocyte distribution, %	12.1 [10.9; 13.3]	11.3 [10.2; 12.4]*	21.0 [18.9; 23.1]*,$^{\#}$

Data are expressed as median and 25th and 75th Percentile. * – significant changes in comparison with control, $p<0.05$; # – significant changes in comparison with hypoxia/reoxygenation, $p<0.05$.

to hypoxia, and its source can be of both endo- and exogenous origin. Considering the role of erythrocyte NO synthase (eNOS) as a source of NO, it is important to note that this enzyme oxidizes L-arginine to NO [23], which is dependent under hypoxic conditions on the partial pressure of oxygen.

A possible reason for the increase in heterogeneity in the SSC value upon molsidomine application is a change in the conformation and packing density of the cytoplasmic and, more likely, the erythrocyte membrane hemoglobin. We have previously shown [16] that hypoxia leads to conformational changes in hemoporphyrin, causing an increase in the packing density of the erythrocyte hemoglobin.

Thus, this experimental study has shown the involvement of the erythrocyte L-arginine-NO-system in adaptation to hypoxia/reoxygenation. The data obtained and the assessment of the influence of NO on the structural and functional organization of erythrocytes under conditions of hypoxia/reoxygenation suggest that the increase in the intracellular concentration of NO under hypoxic conditions is provided by nitrites in the extracellular fluid. The process of adaptation to hypoxia/reoxygenation includes a change in the affinity of hemoglobin for oxygen under conditions of the increased anisocytosis, the increased volume and surface area of erythrocytes.

CONCLUSIONS

Hypoxia modeling (hypoxia simulation) is characterized by an increase in the affinity of hemoglobin for oxygen, while reoxygenation is characterized by a decrease in this parameter. The use of an NO donor under these conditions does not change the affinity of hemoglobin for oxygen; this suggests the depletion of this adaptation mechanism.

The increase in the intracellular NO concentration under conditions of hypoxia/reoxygenation is provided by nitrites in the extracellular fluid: this is confirmed by negative correlations between these parameters. The use of molsidomine under conditions of hypoxia/reoxygenation is accompanied by a higher increase in NO without involvement of the nitrite reductase mechanism.

Adaptive reactions of erythrocytes under conditions of hypoxia/reoxygenation modeling are manifested by changes in the size and densitometric properties of erythrocytes. The use of the NO donor leads to an increase in the average volume of erythrocytes, anisocytosis, and an increase in the cell surface area.

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COMPLIANCE WITH ETHICAL STANDARDS

The study was approved by the Ethics Committee of the National Anti-Doping Laboratory (protocol No. 065/19 of December 27, 2019).

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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ВКЛАД ГАЗОТРАНСМИТТЕРА МОНООКСИДА АЗОТА В СТРУКТУРНО-ФУНКЦИОНАЛЬНУЮ ОРГАНИЗАЦИЮ ЭРИТРОЦИТОВ В УСЛОВИЯХ ГИПОКСИИ/РЕОКСИГЕНАЦИИ

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Гипоксия сопровождается изменениями в метаболизме и функции клеток. Гемоглобин эритроцитов может принимать участие в адаптации к гипоксии, выступая сенсором кислорода, обеспечивая взаимосвязь между содержанием кислорода и кровотоком. Механизмы, обеспечивающие эту функцию, окончательно не установлены. В данной работе исследовали влияние NO на структурно-функциональную организацию эритроцитов в условиях гипоксии/реоксигенации. Установлено, что NO принимает участие в адаптивных реакциях при моделировании гипоксии/реоксигенации, меняя конформацию гемоглобина, что обуславливает изменение спектральных характеристик гемопротеина и сродства гемоглобина к кислороду на фоне роста анизоцитоза, увеличения объёма и площади поверхности клеток. Увеличение внутриклеточной концентрации NO в условиях гипоксии обеспечивается за счёт нитритов внеклеточной жидкости. Молсидомин (донор NO) при моделировании гипоксии/реоксигенации вызывает более высокий прирост NO без включения нитритредуктазного механизма, приводит к росту среднего объёма эритроцита, анизоцитозу, увеличению площади клеточной поверхности.

Полный текст статьи на русском языке доступен на сайте журнала (http://pbmc.ibmc.msk.ru).

Ключевые слова: эритроциты; гипоксия/реоксигенация; монооксид азота; проточная цитометрия; спектроскопия; молсидомин

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