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Potential role of cytoplasmic protein binding to erythrocyte membrane in counteracting oxidative and metabolic stress

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The ability of protein to reversibly bind with membrane components is considered to be one of the oldest mechanisms of cell response to external stimuli. Erythrocytes have a well-developed mechanism of an adaptive response involving sorption-desorption processes, e.g. interactions of key glycolytic enzymes and hemoglobin with band 3 protein. A few publications have shown that under oxidative stress, cytoplasmic enzymes such as catalase, glutathione peroxidase and peroxiredoxin bind to the erythrocyte membrane. The present work is a continuation of research in this direction to determine the causes and consequences of the interaction of cytoplasmic proteins with the membrane under conditions of oxidative stress and different glucose content. Human erythrocytes were incubated for five hours at 20 °C in an oxidizing medium of AscH – $1 \cdot 10^{-4}$ M, Cu^{2+} – $5 \cdot 10^{-6}$ M with different glucose content (0–8 mM). Dynamic changes in the accumulation of membrane-bound hemoglobin, the distribution of ligand forms of hemoglobin in the cytoplasmic and membrane-bound fractions, the activity of membrane-associated and cytoplasmic forms of Cu/Zn superoxide dismutase (SOD1) and catalase, H_2O_2 content in extracellular and intracellular media were recorded. It was shown that binding of catalase and SOD1 to the erythrocyte membrane is initiated by oxidative stress and is a physiological function aimed at complete inactivation of extracellular and H_2O_2 and protection against their entry into the cell. It was shown that under conditions of glucose depletion and oxidative loading, catalase and SOD1 bind to the erythrocyte membrane, leading to inactivation of these enzymes. Membrane-bound hemoglobin was higher in cells incubated under these conditions than in glucose experiments. Glucose introduced into the incubation medium in an amount 4–8 mM causes complete binding of SOD1 to the membrane of erythrocytes, by involving it in the processes of casein kinase stabilization and glycolytic fluxes regulation. With mild oxidation, the amount of hemoglobin bound to the membrane does not change, indicating the presence of certain binding sites for hemoglobin with membrane proteins. We show that the activity of membrane-bound SOD1 along with the content of ligand forms in the composition of membrane-bound hemoglobin are informative indicators of the metabolic and redox state of erythrocytes.

Keywords: oxidative stress; catalase; superoxide dismutase; ascorbate; copper ions; ligand forms of hemoglobin; membrane-bound hemoglobin.

Introduction

The human erythrocyte (RBC) membrane plays a key role in providing and regulating the physiological activity of these cells. It is a complex structure composed by a lipid bilayer and cytoskeleton proteins tethered together by transmembrane proteins such as band 3 protein (CDB3) and glycoporphins. Enzymes bound to the cytoplasmic surface of the RBC membrane may represent an extension of membrane organization into the cytoplasm. The ability of proteins to bind back to membrane components is considered to be one of the oldest mechanisms of response to external influences (Kosmachevskaya et al., 2019). As a rule, the binding of enzymes to membranes leads to a decrease in their activity and is directly dependent on the energy charge of the cell. By binding enzymes, the cell can change its metabolism in seconds, while the realization of the response via changes in gene expression is more time consuming (Kosmachevskaya et al., 2019). RBCs have a well-developed mechanism for forming an adaptive response involving sorption-desorption processes. An example of reversible binding is the interaction of key glycolytic enzymes with CDB3 (Puchulu-Campanella et al., 2013; Chu et al., 2016; Kosmachevskaya et al., 2019). Aldolase and phosphofructokinase and glyceraldehyde-3-phosphate dehydrogenase are enzymes associated with band 3 protein and other membrane proteins (Puchulu-Campanella et al., 2013). These enzymes, as well as pyruvate kinase and lactate dehydrogenase form a

multi-enzyme complex. Hemoglobin in the deoxygenated state has a high affinity for CDB3 (D'Alessandro et al., 2015; Sega et al., 2015; Kosmachevskaya et al., 2019; Zhou et al., 2019). An increase in the proportion of deoxyhemoglobin (deoxyHb) under hypoxia leads to the displacement of glycolysis enzymes from the CDB3 binding site and their transition to a soluble active state (D'Alessandro et al., 2015; Kosmachevskaya et al., 2019). It is believed that the redistribution of hemoglobin (Hb) between soluble and membrane-bound forms switches metabolism from the pentose phosphate pathway to the glycolytic in response to changes in cell oxygen status (D'Alessandro et al., 2015; Sega et al., 2015; Andreyeva et al., 2019). On the one hand, it increases the level of intracellular ATP, and, on the other hand, reduces the glucoses flux that enter the pentose phosphate pathway, which produces NADPH, necessary for the work of antioxidant enzymes, and reduced glutathione. Because CDB3 is the center of erythrocyte membrane organization and the regulator of ionic homeostasis, even minor changes in CDB3 binding lead to changes in the morpho-biochemical properties of cells (Ugurel et al., 2020).

As oxygen-carrying cells, RBCs are repeatedly at risk of oxidative damage (Cortese-Krott & Shiva, 2019). In fact, about 3% of hemoglobin undergoes autooxidation during oxygen dissociation to form oxidized Hb and superoxide anion radical, which after dismutation forms hydrogen peroxide (H_2O_2) (Ratanasopa et al., 2015; Rocha et al., 2015; Kuhn et al., 2017). Oxidation of hemoglobin by H_2O_2 can cause structural modifica-

tions of the globin molecule (Lin et al., 2015; Ratanasopa et al., 2015; Rocha et al., 2019). Oxidation of cysteine residues leads to partial breakdown of its β -chain. Membrane rearrangement in response to oxidants (Boulet et al., 2018) can affect the structure of a protein (be a denaturing agent) by enhancing electrostatic interactions near its surface, namely, by the combined action of local pH reduction and dielectric constant (Bychkova et al., 2014). Thus, oxidized hemoglobin, especially its low-spin forms (hemichrome), have an increased ability to bind to the membrane. Additional ways of binding cytoplasmic proteins to the erythrocyte membrane, such as covalent binding through disulfide bonds and hydrophobic interactions, may occur during the interaction. It has been shown that the oxidation state of hemoglobin (from Fe(III) to Fe(V)) correlates with the binding intensity, with approximately 50% of this binding requiring reactive sulfhydryl groups (Welbourn et al., 2017). Methemoglobin (MetHb), which is not reduced by membrane-bound methemoglobin reductases, degrades to hemichromes (HemiCr) that accumulate in the membrane forming Heinz bodies, causing aggregation of band 3 protein and additional membrane remodeling. Increased amounts of membrane-bound Hb have recently been associated with many diseases of the blood system caused by antioxidant disorders and the action of various xenobiotics (Carelli-Alinovi & Misi, 2017; Welbourn et al., 2017; Kosmachevskaya et al., 2019; Tharoux, 2019).

Catalase (CAT), glutathione peroxidase (GPx) and peroxiredoxin (Prx2) are involved in preventing the accumulation of H_2O_2 in RBC by converting H_2O_2 to water and O_2 . CAT, GPx and Prx2 are essentially cytosolic enzymes; however, the association of these enzymes with the erythrocyte membrane has been reported in different *in vivo* and *in vitro* studies (Bayer et al., 2016; Melo et al., 2019; Rocha et al., 2019). The authors of these studies believe that the binding of these cytosolic enzymes to the membrane is caused by metabolic stress, possibly to protect the erythrocyte membrane and counteract the effects of oxidative stress. Catalase has been shown to retain its activity upon membrane binding (Aviram & Shaklai, 1981), while GPx and Prx2 not (Rocha et al., 2015).

Superoxide dismutase (CuZnSOD, SOD1) is another fundamental antioxidant enzyme that is the only form of SOD in mature mammalian erythrocytes. Erythrocytes deficient in this enzyme have been shown to have a shorter lifespan due to increased levels of reactive oxygen species, lipid peroxidation products, increased hemoglobin susceptibility to oxidation, and Heinz bodies formation (Grzelak et al., 2009). It has been shown that under conditions of normoxia, membrane-bound SOD1 compounds form 0.1% of the total mass of membrane proteins. Under conditions of hypoxia, its content increases 1.5 times (Sidorenko et al., 2018).

In connection with the above, we continue to study the effect of the radical-generating system Asc-Cu²⁺ on erythrocytes in suspension with different glucose content. We study the effect of glucose and mild oxidative environment on the accumulation dynamics of membrane-bound hemoglobin, the distribution of ligand forms in its cytoplasmic and membrane-bound fractions. We determine the activity of membrane-associated and cytoplasmic forms of the enzymes SOD1 and catalase and evaluate the levels of H_2O_2 in the extracellular environment and in erythrocytes. Analyzing the experimental data, we try to explain the interaction of cytoplasmic proteins with the membrane caused by oxidative stress and glucose.

Materials and methods

The protocol of the experimental part was corresponded to the principles of biological ethics and was agreed upon with the Local Ethics Committee of the Vasyl' Stus Donetsk National University, Faculty of Chemistry, Biology and Biotechnology (Vinnitsa, Ukraine).

Peripheral blood of healthy donors of one sex and about one age was used in the study. Erythrocytes were washed three times with centrifugation in Na-phosphate buffer (0.015 M, pH 7.4), containing 0.15 M of NaCl (buffer solution 1). Plasma was washed out and packed erythrocytes were resuspended in the same buffer with different glucose content. The amount of glucose was added in correspondence to the quantity of packed erythrocytes in the suspension to the final concentration range 0, 0.5, 2, 4, 8 mM per 10^{12} erythrocytes/L. The erythrocyte suspension was introduced into the oxidizing medium of the following composition: as-

corbic acid (AscH) – $1 \cdot 10^{-4}$ M, Cu²⁺ – $5 \cdot 10^{-6}$ M, Na-phosphate buffer (0.015, 0.15 M NaCl, pH 7.4) with 5 hours incubation at 20 °C. The number of erythrocytes in the incubation medium was maintained at a level corresponding to 3.0–3.2 mg/mL hemoglobin content. After selected intervals, the sample was washed by centrifugation with Na-phosphate buffer (pH 7.4), after which the washed erythrocytes were resuspended in the original volume of the same buffer.

For further studies (1) erythrocytes washed from the oxidizing medium in a buffer solution 1, (2) hemolysate of erythrocytes washed from the oxidizing medium, (3) erythrocytes ghosts were used.

Hemolysate for catalase and SOD1 activity determination was obtained by adding 0.3 mL of 0.02% saponin solution in 0.01 M Na-phosphate buffer (pH 7.4) to 1 mL of erythrocyte suspension. Hemolysis was performed in cold for 10 minutes.

As control, erythrocytes that were not exposed to radical-generating systems and were in buffer solution 1 with the appropriate amount of glucose were used. The activity of membrane-bound enzymes was determined using whole erythrocytes, the activity of the cytoplasmic fraction was determined by the difference between the activity of hemolysates and the activity recorded on the cell surface.

Superoxide dismutase activity was estimated on the basis of the inhibition of the adrenaline autooxidation in an alkaline medium (carbonate buffer) (Sirota, 1999; Grzelak et al., 2009). The oxidation product of adrenaline has an absorption in the region of 347 nm, its formation occurs in the absence of additional generation factors and is sensitive to SOD1. The magnitude of SOD1 activity in hemolysates and whole erythrocyte cells was judged by the degree of enzyme inhibition by the rate of adrenaline autooxidation. SOD1 activity was counted on the amount of hemoglobin in the sample (mg).

Catalase activity was determined by the rate of utilization of hydrogen peroxide (H_2O_2). The amount of H_2O_2 was determined using a Fox reagent (Wolff & Dean, 1987). The activity of the enzyme was expressed as the μ M of substrate (H_2O_2), converted by the enzyme per min, per mg of hemoglobin (Hb) in the sample.

The H_2O_2 content in the extracellular medium was determined using Fox-reagent, after cell precipitation by centrifugation. The cytosol H_2O_2 content was determined in lysate obtained by subsequent lysis of cells in 0.5 mL of cold water and precipitation proteins by TCA acid (Wolff, 1994; Bou et al., 2008).

The content of membrane-bound hemoglobin and its ligand forms were assessed after cells incubation for 5 hours in Cu²⁺-Asc medium without and in presence of glucose at concentrations of 4 and 8 mM per of 10^{12} of cells/L.

At certain intervals, erythrocytes were lysed by adding 5 (7) mL of 0.01 M Na-K-phosphate buffer (pH 7.4) T = 4 °C. The ghosts were precipitated by centrifugation (10 min, 3000 rpm), the total hemoglobin content and content of ligand forms of hemoglobin in the supernatant was examined. Erythrocyte ghosts were washed with buffer 1 and dissolved in 0.5 (0.2) mL of 5% Triton X-100 solution (Ratanasopa et al., 2015; Rocha et al., 2019). The sample was kept for 5 min until complete clarification of the solution and then buffer 1 was added.

The absorption spectra of cytoplasmic and membrane-bound hemoglobin of erythrocytes were recorded in the wavelength range 500–700 nm in cuvettes with a thickness of 1 mm. A solution containing 0.5 (0.2) mL of 5% Triton X-100 and buffer solution 1 was used as a reference solution for spectrophotometric measurements to study of membrane-bound hemoglobin. The total content of cytoplasmic and membrane-bound fractions of hemoglobin was determined by absorption at a wavelength 223 nm using an extinction coefficient of 7120 M/cm (Ratanasopa et al., 2015).

To determine the content of ligand forms of hemoglobin, the absorption at 540, 560, 576 and 630 nm (Attia et al., 2019) was recorded. The content (C) of ligand forms of hemoglobin (in M) was calculated using the equations given in (Benesch et al., 1973):

$$C_{OxyHb} = (1.4747 \cdot A_{576} - 0.6820 \cdot A_{560} - 0.5329 \cdot A_{540}) \cdot 10^{-4},$$

$$C_{MetHb} = (4.5852 \cdot A_{540} - 0.8375 \cdot A_{560} - 3.7919 \cdot A_{576}) \cdot 10^{-4},$$

$$C_{deoxyHb} = (1.4749 \cdot A_{560} + 0.2141 \cdot A_{576} - 1.1042 \cdot A_{540}) \cdot 10^{-4},$$

$$C_{HemiCr} = ((1.8787 \cdot A_{560} - 1.4061 \cdot A_{576} - A_{630}) / 8.6888) \cdot 10^{-4},$$

where A_{540} , A_{560} , A_{576} , and A_{630} are the absorbances measured experimentally at respective wavelengths for experimental Hb solutions (Attia et al.,

2015). The content of each form was expressed as a percentage of the total hemoglobin content in the cytoplasmic (membrane-bound) fraction. The total hemoglobin content in erythrocytes was determined by the hemoglobin cyanide unified method according to the standard set at a wavelength 540 nm.

All experiments were performed in five replicates. The data were analyzed in the Statistica 8.0 (StatSoft Inc., USA) software. The data are presented in the tables as $x \pm m$ (x – mean, m – error). The statistical relationship between the pairs of studied indicators was evaluated using the Spearman correlation coefficient (r). Significance of correlation coefficients ($P < 0.05$) was determined by Student's t -test. To construct the distribution curves of the ligand forms of hemoglobin we used the approximation of data by the method of least squares in accordance with the equation of polynomial regression of the 6th degree.

Results

The H_2O_2 change in erythrocytes (b) and extracellular medium during 5 hours of incubation are shown in Figure 1 with indication of the absolute values of H_2O_2 in the extracellular environment that could be used to compare its amount in the medium without cells. The data obtained indicate the ability of erythrocytes to effectively neutralize extracellular hydrogen peroxide. Glucose at the maximum studied concentration was involved in the production of H_2O_2 , as evidenced by the gradual increase of the latter (H_2O_2 content at the end of the experiment reached $10.9 \pm 1.06 \mu M$).

The relative changes in cytoplasmic hydrogen peroxide are shown in Figure 1b. The baseline level of H_2O_2 , its content in cells before placement in an oxidizing medium was considered. In the absence of glucose in the incubation medium, H_2O_2 gradually accumulated. Intracellular oxidative stress developed 15–20 min after the introduction of erythrocytes into the Cu^{2+} -AsCh system (Fig. 1b) and could be explained by the high activity of membrane-associated SOD1 ($r = 0.86$) and not the flow of hydrogen peroxide into the cell (no correlation). Addition of glucose to the extracellular medium at the concentration 0.5 and 2.0 mm reduced the amount of H_2O_2 in both the extracellular environment and the cytosol of cells. The presence of glucose at concentration 4 and 8 mm contributed to an increase in hydrogen peroxide in the extracellular environment, but in these cases H_2O_2 did not accumulate in the cells themselves, and even at some intervals its level in the cytosol fell below baseline.

The changes of membrane-bound SOD1 in the absolute activity units are shown in Figure 2. In glucose-free medium, during the first three hours of the experiment (0–180 min) the activity of membrane-bound SOD1 increased by 2.3 ± 0.4 . The activity of SOD1 in the cytoplasm (data not shown) decreased by 9.7 ± 1.28 . After 210 min of incubation, the activity of membrane-bound SOD1 decreased rapidly, and at the end of the experiment had lost more than 70% of its activity. The activity of SOD1 in the cytoplasm increased, but was reduced by 4 times from baseline.

Addition of glucose (up to 2 mm) resulted in a smaller increase in the activity of membrane-bound SOD1 relative to the control level, but the activity of the enzyme remained stable throughout the experiment. In the cytoplasm the SOD1 activity remained low. In experiments with a glucose concentration of 4 and 8 mm, the nature of the change in SOD1 activity was different: SOD1 activity gradually decreased by half during 90–150 min from the beginning of the experiment, with subsequent increase, but still remained below the control. However, we were not able to detect SOD1 activity in the cytoplasm.

The change of membrane-bound and cytoplasmic catalase activity in the erythrocytes depending on the level of glucose in the incubation medium is shown in Figure 3. At glucose concentrations of 0–2 mM, the activity of membrane-bound catalase varied in two phases: for 90–120 min it decreased by an average of 20%, then increased to the control level. The rise in glucose concentration in the incubation medium led to an increase in the activity of the membrane-bound enzyme, especially at the end of the experiment. At 4 mM glucose, the activity of membrane-bound catalase was 1.41 ± 0.11 times higher and at 8 mM 1.23 ± 0.06 times higher than the baseline (Fig. 3a). The activity of cytoplasmic catalase under conditions of glucose depletion dropped significantly: after 90 min from the beginning of the experiment by $60.0 \pm 14.7\%$, at the end of the experiment by $80.6 \pm 8.6\%$ (Fig. 3b).

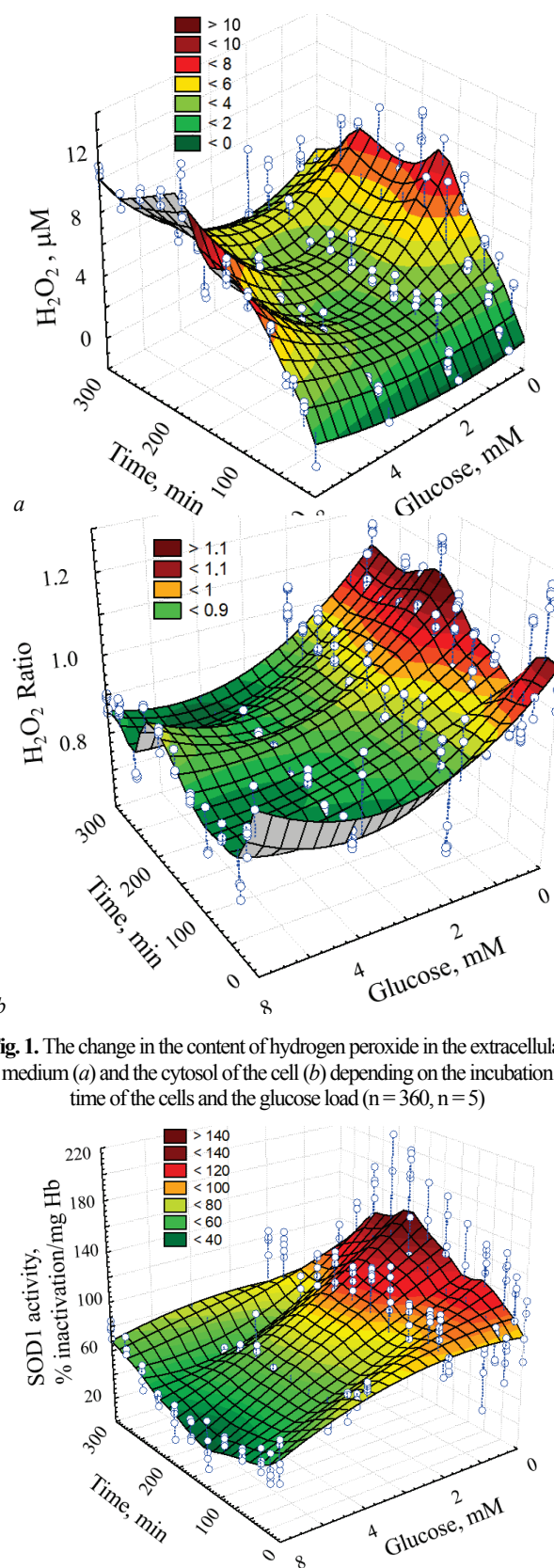


Fig. 1. The change in the content of hydrogen peroxide in the extracellular medium (a) and the cytosol of the cell (b) depending on the incubation time of the cells and the glucose load ($n = 360$, $n = 5$)

Fig. 2. The change in SOD1 activity of erythrocyte surface activity depending on incubation time of cells and glucose load ($n = 180$, $n = 5$)

The introduction of glucose into the incubation medium in the amount of 2 and 4 mM contributed to a significant increase in the activity of cytoplasmic catalase for 150 min of the experiment, after that the catalase activity decreased to the level of control and 30% below control, respectively. At 8 mM glucose concentration an increase in activity was observed at the beginning of the experiment with a gradual decrease in activity by

69.5 ± 1.4%. The correlation coefficient between the activities of membrane-bound and cytoplasmic catalase was -0.77 in experiments with high glucose concentration (4 and 8 mM).

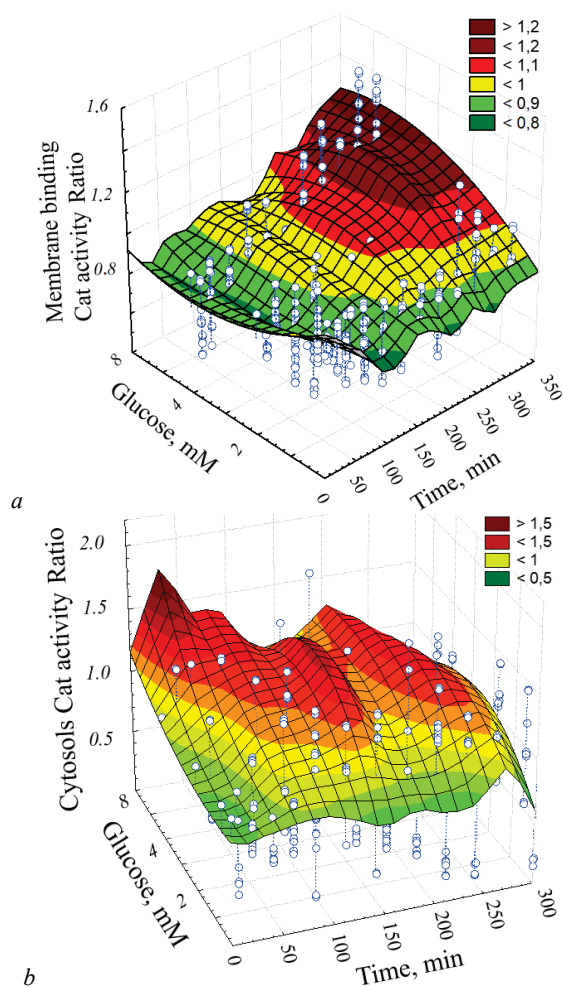


Fig. 3. The change in membrane-bound (a) and cytoplasmic catalase (b) activity depending on the incubation time of the cells and the glucose load ($n = 360, n = 5$)

During incubation the level of membrane-bound hemoglobin reached a certain equilibrium level. The initial level of membrane-bound hemoglobin varied at control points from 1.5 to 10.0% and depended on the conditions of cell secretion and the presence of glucose. After one hour of incubation, equilibrium levels of membrane-bound hemoglobin were established in the cells and maintained until the end of the experiment. In cells incubated without glucose the level of membrane-bound hemoglobin was $4.43 \pm 0.56\%$ of total hemoglobin. In cells incubated in medium with 4 mM glucose it was $2.25 \pm 0.31\%$, and in 8 mM glucose - $3.36 \pm 0.78\%$, but in latter case, during the last hour of incubation, the content of membrane-bound hemoglobin decreased almost twice and was $1.88 \pm 0.08\%$.

The changes in ligand forms of cytoplasmic (a) and membrane-bound (b) hemoglobin in erythrocytes incubated in glucose-free medium are shown in Figure 4. The content of OxyHb in the cytoplasmic fraction during 120 min of incubation decreased to $64.8 \pm 0.78\%$, the amount of methemoglobin increased to $34.6 \pm 1.8\%$ (Fig. 4a). These parameters correlated with the content of hydrogen peroxide in the cells. At the end of incubation, the level of MetHb decreased slightly, and the content of oxyhemoglobin remained at $71 \pm 1.1\%$ (Fig. 4a). The correlation coefficient between OxyHb and MetHb is -0.87 and between DeoxyHb and MetHb was -0.7. In the membrane-bound hemoglobin fraction, the OxyHb content ranged 60–75%, the DeoxyHb content 3–10%, and the MetHb content ranged 20–38%. A significant correlation coefficient (-0.59) was found only between DeoxyHb and MetHb. The hemichrome content in the cell did not exceed 2%.

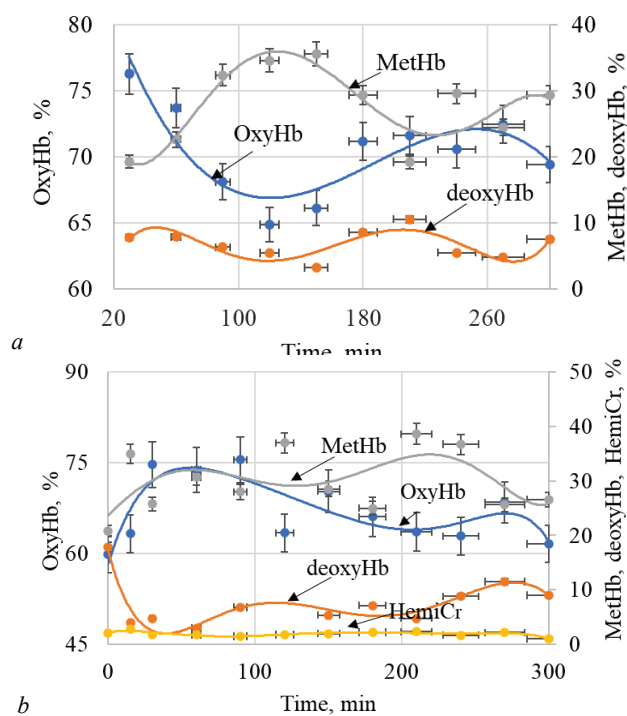


Fig. 4. The distribution of ligand forms in the composition of cytoplasmic (a) and membrane-bound (b) fractions of erythrocytes hemoglobin that were incubated in AscH-Cu^{2+} medium without glucose: each point represents the $x \pm m$ for $n = 5$

The changes in the ligand forms of cytoplasmic (a) and membrane-bound (b) hemoglobin in erythrocytes incubated in a 4 mM glucose medium are shown in Figure 5. The average level of oxyhemoglobin in the cytoplasm was $78.3 \pm 2.4\%$, deoxyhemoglobin $7.0 \pm 2.2\%$, the level of methemoglobin was lower and ranged from $11.3 \pm 0.6\%$ to $22.6 \pm 1.0\%$. The correlation between OxyHb and MetHb in the cytoplasmic fraction was -0.82. In addition, membrane-bound hemoglobin significantly decreased oxyhemoglobin levels due to an increase in deoxyform. After 100 min of incubation, the level of OxyHb in the membrane was $37.4 \pm 0.8\%$, deoxyHb - $23.5 \pm 2.3\%$. The maximum level of methemoglobin $30.8 \pm 2.8\%$ was recorded only at the beginning of the experiment, and later its amount decreased almost twofold. We recorded a slightly elevated level of hemichrome in the membrane-bound form, but its content remained approximately constant (4–5%) throughout the experiment. No significant correlations were found between the ligand forms in membrane-bound hemoglobin, but a correlation was found between the activity of membrane-bound SOD1 and OxyHb ($r = 0.56$) and deoxyHb ($r = 0.76$).

In the cytosol of erythrocytes incubated in a medium with 8 mM glucose (Fig. 6a) the content of OxyHb was $76.8 \pm 3.6\%$, deoxyHb - $8.8 \pm 1.8\%$. MetHb was maintained at $22.1 \pm 2.1\%$ for 120 min, and then began to decline. At the end of the experiment, the MetHb content increased to $25.3 \pm 0.4\%$ of the total amount of cytoplasmic hemoglobin. The distribution of ligand forms in membrane-bound hemoglobin (Fig. 6b) is also very similar, but the decrease in oxyhemoglobin content began only after 120 min of cell incubation. The content of ligand forms in the composition of membrane-bound hemoglobin correlates with the activity of membrane-bound SOD1.

Discussion

Oxidative stress plays an important role in pathogenic conditions, leading to direct cell damage and affecting dozens of metabolic pathways. Cell membranes in general and the erythrocyte membrane in particular are very sensitive to oxidative damage. Various active oxygen species, such as hydroxyl radicals, singlet oxygen, superoxide and H_2O_2 , initiate lipid peroxidation, leading to loss of membrane integrity. Glutathione peroxidase, superoxide dismutase and catalase are involved in erythrocytes' protective mechanisms against the deleterious effects of oxidizing products. Location

of these enzymes in the proximity of the membrane would significantly increase their efficiency in the scavenging of reactive oxygen species.

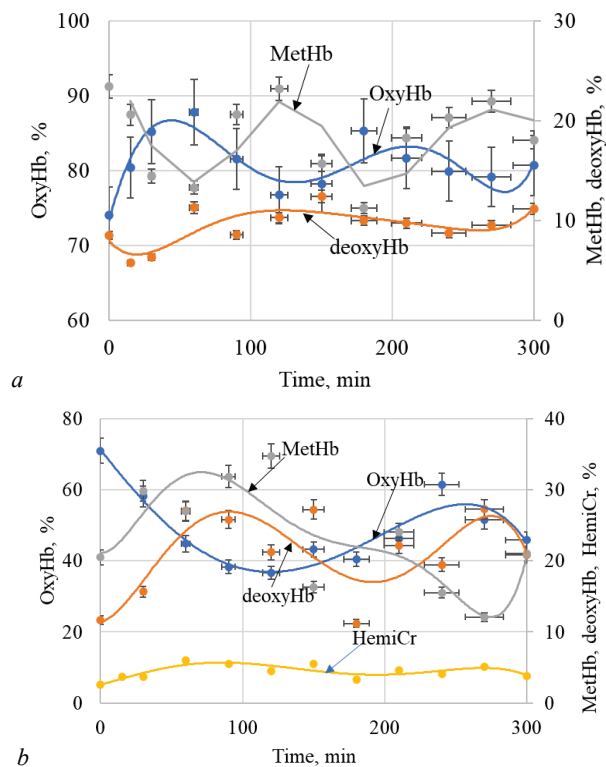


Fig. 5. The distribution of ligand forms in the composition of cytoplasmic (a) and membrane-bound (b) fractions of erythrocytes' hemoglobin that were incubated in AsCh-Cu²⁺ medium with 4 mM glucose: each point represents the $\bar{x} \pm m$ for $n = 5$

It is known that erythrocytes rearrange the membrane under the influence of various external factors (Carelli-Alinovi & Misi, 2017; Boulet et al., 2018), resulting in a violation of the asymmetry of the lipids' location and the charge of the membrane surface. In this case, with a high degree of probability the protein native structure could be transformed under the action of certain internal factors into a state similar to denatured (Bychkova et al., 2014; Cheng et al., 2016). Along with natural denaturing factors, such as low pH, thermal and salt stresses, a negatively charged membrane surface can serve as a moderate denaturing agent in the cell, due to increased electrostatic interactions at the interface between the aqueous and nonpolar phases. As a result, the membrane goes into a state other than the native, which allows the cell to function in a different physiological mode (Dotsenko et al., 2012), as this changes the work of ion transport systems, the inclusion of secondary messengers (especially calcium-mediated regulatory mechanism) that are aimed at restructuring functional state of the cell as a whole.

The obtained data suggest that the oxidative load creates the conditions for the cytoplasmic enzymes binding to the erythrocyte membrane. It is known that catalase can bind to the erythrocyte membrane (Aviram & Shaklai, 1981; Rocha et al., 2015; Melo et al., 2019; Rocha et al., 2019). Binding occurs through electrostatic interactions between the negatively charged membrane and the enzyme (Aviram & Shaklai, 1981), but ionic binding to the membrane involving calcium and sulfhydryl groups (Allen et al., 1977) may also occur. The outer surface of erythrocytes, bearing high negative charge as well, does not bind catalase (Aviram & Shaklai, 1981). In respect to that, catalase resembles other components of the cytosol, such as hemoglobin and a number of glycolytic enzymes which are specifically associated with the inner side of the membrane. Moreover, the fact that catalase binding is not affected by the removal of spectrin and actin from the membrane indicates that the cytoskeletal composition does not prevent occupation of a major part of the membrane region by other proteins (Aviram & Shaklai, 1981). It is believed that the enhanced association of CAT with the membrane is a consequence of metabolic stress caused by destabilization of the membrane structure due to its altered composition and is exacerbated by hematological diseases associated with oxidative stress,

such as hereditary spherocytosis (Allen et al., 1977; Rocha et al., 2019), beta-thalassemia, erythrocyte enzymopathy, blood storage (Rocha et al., 2015; Melo et al., 2019; Rocha et al., 2019).

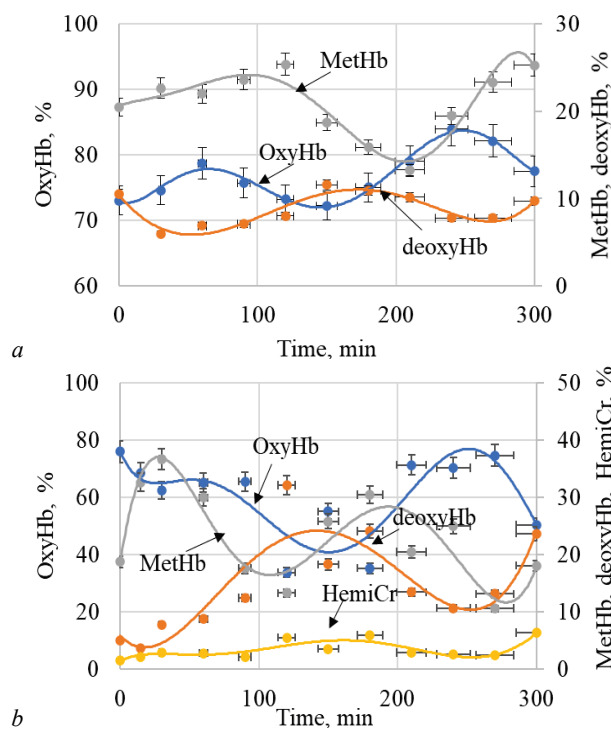


Fig. 6. The distribution of ligand forms in the composition of cytoplasmic (a) and membrane-bound (b) fractions of erythrocytes hemoglobin that were incubated in AsCh-Cu²⁺ medium with 8 mM glucose: each point represents the $\bar{x} \pm m$ for $n = 5$

Under conditions of glucose depletion and, as a consequence, depletion of ATP and NADPH, erythrocytes are forced to rearrange metabolic processes for survival. We show that the activities of membrane-bound catalase and SOD1 in this case correlate with the amount of external hydrogen peroxide (correlation coefficients -0.66 and 0.86 , respectively), that is the transition of enzymes to the bound state is initiated by oxidative stress and H₂O₂ production externally. At the beginning of the experiment, the catalase activity of intact cells and hemolysate coincided. During the 90 min incubation, we recorded a direct correlation (0.78) between the bound and cytoplasmic forms of catalase (the activities of both forms are reduced) and the inverse (-0.68) by the end of the experiment. The fall in cytoplasmic enzyme activity was $80.6 \pm 8.6\%$, while its increase on the surface was only $21.3 \pm 6.0\%$. Catalase converts to a membrane-bound form with activity loss of unknown mechanisms. The deeper location of the heme may be advantageous during its interaction with the membrane when the protein is exposed to phospholipids. The result of this interaction may be the location of the prosthetic group between the lipid and protein phases (Aviram & Shaklai, 1981), which leads to a loss of enzymatic activity. But we do not rule out the possibility of binding catalase in the oligomeric state. Earlier we showed (Taradina & Dotsenko, 2011) that the action of very weak factors leads to denaturation changes in the active center of native catalase, resulting in the formation of various oligomeric intermediates, among which there are partially denatured and dissociated forms with reduced activity, compared with the tetrameric form. Our hypothesis is evidenced (Allen et al., 1977) by the fact of the existence of monomeric catalase without loss of subunit structure, completely inactive form in erythrocytes of patients with hereditary spherocytosis.

In terms of energy, erythrocytes are dependent on the glucose consumption, which is metabolized in the reactions of glycolysis and pentose-phosphate shunt, providing the cell with ATP and reducing equivalents. In the current study we showed that the presence of glucose in the incubation medium affects the behaviour of antioxidant enzymes and their binding to the membrane. At low glucose concentrations (up to 4 mM), catalase passes into the membrane with virtually no loss of activity. Thus, the

activity of cytoplasmic catalase after 5 hours of incubation in solutions with a glucose content of 4 mM is reduced by $30.0 \pm 1.3\%$, while the activity of membrane-bound catalase increases by $26.0 \pm 1.1\%$ (correlation coefficient between the two forms of the enzyme -0.77). Addition of glucose to the oxidizing environment attracts glucose to the formation of hydrogen peroxide. The H_2O_2 content increases in the extracellular environment, especially at the end of incubation. The activity of cytoplasmic catalase of erythrocytes at the end of the experiment in solutions with 8 mM glucose content decreases by $59.5 \pm 1.4\%$ and the activity of membrane-bound catalase increases by $5.8 \pm 1.3\%$. Thus, we have shown that the more H_2O_2 accumulates externally, the more inactivated bound catalase in the membrane is observed (inactivation of H_2O_2 catalase is a known fact). It is believed that extracellular H_2O_2 freely enters the cytoplasm of the cell. Earlier (Dotsenko et al., 2010), we investigated in detail the ability of a system, containing Cu^{2+} ions and ascorbic acid (AscH), to increase the production of hydrogen peroxide. We showed that in the Cu^{2+} -AscH system the content of H_2O_2 increases over time and reaches $43.6 \pm 0.87 \mu M$ five hours from the start of the reaction. Our data suggest that in the presence of erythrocytes, the maximum concentration of H_2O_2 reaches $10.9 \pm 1.06 \mu M$ in the medium only in the presence of glucose in the amount of 8 mM. Clearly, membrane-bound catalase is fully capable of inactivating extracellular H_2O_2 , and membrane-bound glutathione peroxidase (GPx) and peroxiredoxin (Prx2) are involved in these processes (Rocha et al., 2015; Bayer et al., 2016; Melo et al., 2019; Rocha et al., 2019). It has been shown (Aviram & Shaklai, 1981) that the cell permeability barrier for H_2O_2 is responsible for the rate of decomposition of hydrogen peroxide. The dense packing of catalase molecules under saturation conditions promotes the efficient entry of H_2O_2 into the active site, where it is consumed at a rate of $3.5 \pm 1.7 M^{-1} s^{-1}$ (Bonaventura et al., 1972). According to our results, no correlations have been established between cytoplasmic and extracellular H_2O_2 content, which indicates the formation of intracellular H_2O_2 due to cellular metabolic processes.

Under conditions of glucose depletion, there is an inverse correlation between cytoplasmic and membrane-associated forms of SOD1 (-0.66). In contrast to catalase, the activity of membrane-bound SOD1 during the 180 min experiment increased significantly, which is unexpected for this enzyme, which is very sensitive to oxidative damage. The recorded changes in the activity of SOD1 on the surface and in the cytoplasm suggest that the binding of the enzyme occurs in a partially denatured state with loss of activity. The increase in the recorded activity of SOD1 may be explained by an increase in the number of membrane-associated forms of protein (Sidorenko et al., 2018). SOD1 has a stable intra-subunit disulfide bond (Cys58-Cys147), which is a rare structural motif in proteins involved in redox transformations. The disulfide bond of Cys58-Cys147 is maintained even if Cys147 is oxidized, and this allows SOD1 to stay in a partially active state for some time (Tiwari et al., 2019). According to these authors, the oxidative modification of SOD1, including the oxidation of a key disulfide bond, can disrupt the structural and functional properties of this enzyme, facilitating the transition of the protein to the denatured state, and this can promote binding to the membrane. After 180 minutes of the experiment, we recorded a sharp decrease in the activity of membrane-bound SOD1, which oxidizes and denatures in the membrane. The data obtained do not support the inverse mechanism of enzyme binding. It is known that SOD1 enzymopathy, associated with the formation of an unstable enzyme, is considered one of the causes of amyotrophic lateral sclerosis, as the oxidation of SOD1 can form amyloid structures (Tiwari et al., 2019).

The dependences of the change in the activity of SOD1 under the presence of glucose in the incubation medium are radically different. We recorded a gradual decrease in the activity of membrane-bound SOD1 when glucose was introduced into the incubation medium. At glucose concentrations of 4 and 8 mM, all SOD1 was in membrane-bound state, its activity decreased for 150–180 min from the beginning of the experiment, and only then grew. This indicates additional variants of SOD1 binding with the membrane, rather than activation by glucose alone. It is known that SOD1 transmits signals from oxygen and glucose to repress respiration (Reddi & Culotta, 2013). The mechanism involves SOD1-mediated stabilization of two homologs of 1-gamma (CK1 γ) casein kinase, Yck1p and Yck2p, required to inhibit oxygen binding. SOD1 binds the C-terminal degron to Yck1p/Yck2p, and promotes kinase stability by catalyzing

the conversion of superoxide to peroxide (Reddi & Culotta, 2013). H_2O_2 plays a role in signaling by activating intracellular tyrosine kinases. In this case, the specific oxidation of sulfhydryl groups of cysteine causes conformational changes that lead to kinase activation (Wang et al., 2018). The effect of SOD1 on the stability of CK1 γ is observed in mammal and human cell lines. In a single circuit, oxygen, glucose, and reactive oxygen can suppress respiration through SOD1/CK1 γ signaling. This mechanism is not described for erythrocytes, but we believe that the binding of SOD1 to casein kinase causes a decrease in enzyme activity. This is indicated primarily by the presence of serine/threonine casein kinases of type I and II in erythrocytes. Casein kinase I phosphorylates CDB3, regulates the structural properties of the erythrocyte membrane (Wang et al., 1997). Casein kinase II is a common regulator of intracellular signaling and metabolism, phosphorylating a wide range of proteins. In erythrocytes, casein kinase II has a modulating effect on transmembrane electron transport (Iakovenko et al., 2012). Secondly, we show a correlation between the activity of SOD1 with the content of intracellular hydrogen peroxide (0.52) and with ligand forms of membrane-bound hemoglobin – OxyHb (0.53), deoxyHb (-0.75), HemiCr (-0.77). The content of deoxyHb in the composition of membrane-bound hemoglobin increases significantly (Fig. 5b). It is known that an increase in the proportion of deoxyHb under hypoxia leads to the displacement of glycolysis enzymes from the binding site to CDB3 and their transition to a soluble active state (D'Alessandro et al., 2015; Chu et al., 2016; Andreyeva et al., 2019; Kosmachevskaya et al., 2019), the metabolic flow is directed to the glycolytic pathway, which allows the production of ATP. During this time, the pentose phosphate pathway of glucose metabolism is inhibited and glycolysis is activated, which reduces the ability of erythrocytes to synthesize NADPH and reduce glutathione, which in turn are required for the reduction of dehydroascorbate to ascorbate as one of the electron transport donors (Tu et al., 2017). In this case, the electron transport chain uses glycolytic NADH as the main electron donor.

The transition from Hb from soluble to membrane-bound state can be both physiological and destructive. In the case of reversible interaction with membranes, the spatial redistribution of Hb enables the level of cellular metabolism to change, which is especially important for erythrocytes, which lack the apparatus of biosynthesis (Rifkind & Nagababu, 2013; Kosmachevskaya et al., 2019). The formation of membrane-bound hemoglobin may be one of the mechanisms for the formation of a rapid adaptive response to changing conditions. As a hemoprotein, hemoglobin itself is able to generate a hydroxyl radical in the presence of H_2O_2 . The consequence of the involvement of hemoglobin in redox reactions is the modification of amino acid residues of different nature (Lin et al., 2019). Covalent modification of proteins affects various biological processes, including activation or inactivation of protein functions. It is believed that modified methemoglobin, which is unable to be reduced by methemoglobin reductases, may bind to the membrane to form hemichrome. The level of hemichrome in cells incubated under conditions of glucose depletion is $1.92 \pm 0.42\%$. In the presence of glucose we record a slightly higher content of hemichrome in the composition of membrane-bound hemoglobin (up to 5%). The content of all three ligand forms of hemoglobin (OxyHb, deoxyHb, MetHb) correlate with each other within one fraction, and there are no links between fractions. This suggests that the transformations involved in membrane-bound hemoglobin occur in the membrane itself (Welbourn et al., 2017). Bound hemoglobin during the study was fully functional, able to react with oxygen and form a functional complex. Bound hemoglobin is also oxidatively active, the oxyform can be autoxidized to methemoglobin, which in turn can be reduced by both membrane-bound reductases and cell reductants. The fact that deoxyform was not accumulating indicates the possibility of its oxygenation. Methemoglobin is functionally active and a decrease in its content is not associated with an increase in hemichrome content in cells.

Conclusion

Binding of catalase and SOD1 to the erythrocyte membrane is initiated by oxidative stress and is a physiological function aimed at complete inactivation of extracellular and H_2O_2 and protection against their entry

into the cell. Binding of these enzymes to the membrane is accompanied by their partial or complete inactivation.

Under conditions of hypoxia, SOD1 is involved in the transmission of signals from oxygen and glucose to regulate intracellular metabolic processes and ATP production. In this case, the binding of SOD1 is regulatory in nature and is a tool for adjusting the properties of the membrane and carbohydrate metabolism under oxidative stress.

With mild oxidation, the amount of hemoglobin bound to the membrane does not change, indicating the presence of certain binding sites for hemoglobin with membrane proteins. The study of the distribution of ligand forms in membrane-bound hemoglobin can be an informative tool for analyzing the state of cells.

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