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Membrane effects of nitrite-induced oxidation of human red blood cells

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Abstract

The aim of our investigation was to study the red blood cell (RBC) membrane effects of $NaNO_2$-induced oxidative stress. Hyperpolarization of erythrocyte membranes and an increase in membrane rigidity have been shown as a result of RBC oxidation by sodium nitrite. These membrane changes preceded reduced glutathione depletion and were observed simultaneously with methemoglobin (metHb) formation. Changes of the glutathione pool (total and reduced glutathione, and mixed protein-glutathione disulfides) during nitrite-induced erythrocyte oxidation have been demonstrated. The rates of intracellular oxyhemoglobin and GSH oxidation highly increased as pH decreased in the range of 7.5–6.5. The activation energy of intracellular metHb formation obtained from the temperature dependence of the rate of HbO_2 oxidation in RBC was equal to 16.7 ± 1.6 kJ/mol in comparison with 12.8 ± 1.5 kJ/mol calculated for metHb formation in hemolysates. It was found that anion exchange protein (band 3 protein) of the erythrocyte membrane does not participate significantly in the transport of nitrite ions into the erythrocytes as band 3 inhibitors (DIDS, SITS) did not decrease the intracellular HbO_2 oxidation by extracellular nitrite.

Keywords: Erythrocyte; Membrane; Nitrite; Oxidative stress; Methemoglobin

1. Introduction

The oxidation of erythrocytes by different agents can be used as a model for the oxidative damage of red blood cell (RBC) functions during their long journey in the blood stream. There are many studies designed to assess the cellular effects of $NaNO_2$ in erythrocytes [1–5]. Nitrite, a well known methemoglobin (metHb)-forming agent [6], is commonly present, for example, as a food contaminant. NO_2^- is the major oxidant product of pure $NO^\bullet$ gas as well as such NO donors as *S*-nitroso adducts of bovine serum albumin or *S*-nitroso adduct of glutathione in phosphate buffer (pH 7.4) [7]. In the presence of oxyhemoglobin (oxyHb) these NO donors, as well as NO donors used in therapy, produce only NO_3^- [7]. Vice versa, non-enzymatic production of nitric oxide from sodium nitrite in the blood of rats was demonstrated [8]. The 6- and 5-coordinated iron complexes between NO and hemoglobin (nitrosylhemoglobin) were detected in the blood of rats treated with sodium nitrite. The same two types of complexes were observed in rat blood treated in vivo and in vitro with nitroglycerin [8].

It was shown that dietary nitrate (a natural component of fruit and vegetables, drinking water) is

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enzymatically reduced to nitrite by bacteria in the oral cavity [9]. The salivary nitrite formed is thought to protect both the oral cavity and the gastrointestinal tract against infectious diseases [9].

Nitrite and nitrate levels in plasma increased as a result of nitric oxide metabolism after NO donor administration [10]. Nitrite is a reliable marker of NO synthase activity. Basal levels for nitrate and nitrite in human blood were determined to be 25 ± 4 μmol/l and 578 ± 116 nmol/l, respectively [11].

It has been reported that the administration of $NaNO_2$ to mice leads to an increase in the metHb level, the activity of glutathione reductase and glucose 6-phosphate dehydrogenase of erythrocytes, and to a decrease in the activity of the key enzymes of antioxidant protection of erythrocytes, superoxide dismutase and catalase. A high level of erythrocyte lipid peroxidation after nitrite intoxication was observed [12]. Sodium nitrite at a dose of 30 mg/kg body weight per day increased metHb concentration by 4.2% after 90 days of intoxication, and decreased free sulfhydryl groups and tryptophan levels in the blood of rats [13]. Ogata et al. [2] reported that the formation of metHb from oxyHb in the blood of mice receiving sodium nitrite appeared to be controlled by the blood catalase.

It has been shown that nitrite treatment of intact RBC causes a noticeable oxidation of oxyHb to metHb by radical generation [1,14–16] and a decrease in the glutathione level in the intracellular medium associated with membrane lipid peroxidation [1]. The oxidative reactivity induced by nitrite alters the cell ionic flux [1].

Nitrite treatment of intact RBC was found to produce almost complete oxyHb conversion to metHb, but no detectable lipid extraction from the membrane into the cytosol [17]. Ogawa et al. [3] demonstrated that oxyHb of canine erythrocytes with inherited high Na,K-ATPase activity was better protected against nitrite-induced oxidation by glutathione excess and by the increase in glycolysis. 3-Ribosyluric acid prevented the oxidation of oxyHb by nitrite in bovine erythrocytes, inhibiting the autocatalytic phase of the reaction [5]. It was shown that diabetic intact erythrocytes were significantly less sensitive compared with those of non-diabetics to oxyHb oxidation caused by nitrite, probably due to differences in the respective cell cytosolic anti-oxidant systems [4].

Recently, nitrite, a major end-product of $NO^{\bullet}$ metabolism, was demonstrated to react with the inflammatory mediators hypochlorous acid (HOCl) or myeloperoxidase, forming nitryl chloride (NO_2Cl) and nitrogen dioxide ($NO_2^{\bullet}$). So, NO_2^- may play a role in phagocyte-mediated oxidative reactions at sites of inflammation and infection [18].

The earlier studies dealt with the intracellular effects of nitrite. At the same time a complex study of the cellular and membrane effects of nitrite ion is lacking. The aim of our investigation was to follow the erythrocyte membrane alterations induced by $NaNO_2$, as well as the role of erythrocyte membrane anion exchanger in the nitrite transport into the cell.

2. Materials and methods

2.1. Chemicals

Reagents of analytical grade were from POCh (Gliwice, Poland). 5,5′-Dithiobis(2-nitrobenzoic acid) (Ellman's reagent), 2-thiobarbituric acid (TBA), trichloroacetic acid (TCA), *tert*-butyl hydroperoxide (*t*-BHP) and valinomycin were from Sigma-Aldrich GmbH, Germany; the fluorescent probes 1-(trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene, *p*-toluenesulfonate (TMA-DPH) and 3,3′-dipropylthiadicarbocyanine iodide (DiS-C_3(5)) were from Molecular Probes, Eugene, OR, USA; 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (disodium salt) (DIDS) was from Sigma, USA, 4-acetamido-4′-isothiocyano-stilbene-2,2′-disulfonic acid (SITS) was from Pierce, USA.

2.2. Blood samples

Blood from healthy donors was purchased from the Central Blood Bank in Lodz. Blood was taken into 3% sodium citrate. After removing plasma and the leukocyte layer, erythrocytes were washed three times with cold (4°C) phosphate buffered saline (PBS: 0.15 M NaCl, 1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4). Erythrocytes were used immediately after isolation.

2.3. The susceptibility of erythrocytes to nitrite-induced damage

Suspensions of RBC in PBS (hematocrit 10%) were treated with different concentrations of sodium nitrite at 37°C for 30 min. The cells were then immediately washed twice with an excess of cold PBS and placed on ice.

In some experiments the hemolysates of the same number of cells in 0.005 M phosphate buffer, pH 7.4 were treated with NO_2^-.

The susceptibility of erythrocytes to oxidative damage was measured in terms of oxidation of intracellular oxyHb and reduced glutathione (GSH), changes of red blood cell membrane fluidity, membrane potential and cell hemolysis.

2.4. The TBARS assay

The amount of TBA-reactive species (TBARS) formed was measured using the method of Stocks and Dormandy [19], modified by Jain et al. [20].

2.5. The concentration of metHb

The concentration of metHb in treated erythrocytes was measured from visible spectra of RBC hemolysates by optical density assay at 500, 576 and 631 nm using extinction coefficients of oxyHb $\varepsilon = 14.37$ mM^{-1} cm^{-1} (576 nm) and metHb $\varepsilon = 3.7$ mM^{-1} cm^{-1} (631 nm) or $\varepsilon = 10$ mM^{-1} cm^{-1} (500 nm) (per heme) [21].

2.6. The concentration of GSH, total glutathione (GSSG+GSH) and protein-glutathione (GSSP) mixed disulfides

The intracellular GSH level was determined by the method of Ellman [22]. Briefly, 0.1 ml of 25% TCA was added to 0.5 ml of RBC suspension and centrifuged. To 50 μl of the supernatant 1 ml of 100 mM phosphate buffer (pH 8.0) and 20 μl of Ellman's reagent (5×10^{-3} M) were added for GSH determination. The concentration of GSH was monitored spectrophotometrically at 412 nm using the extinction coefficient of 13.6 mM^{-1} cm^{-1}. Total glutathione in RBC was determined enzymatically in a recycling assay using glutathione reductase, NADPH and Ellman's reagent according to the procedure of Akerboom and Sies in the acid-soluble fraction of the cells [23]. Mixed protein-glutathione disulfide (PSSG) were estimated according to Rossi et al. [24].

2.7. Steady-state fluorescence anisotropy

Steady-state fluorescence anisotropy measurements of the TMA-DPH probe incorporated into RBC membranes were performed at room temperature with a Perkin-Elmer LS-5B spectrofluorimeter. Prior to anisotropy measurements samples of red blood cells were diluted to a hematocrit of 0.05% to avoid depolarization effects due to light scattering. The final concentration of the fluorescent probe in samples was 1 μM, $\lambda_{exc} = 356$ nm and $\lambda_{em} = 428$ nm. The steady-state fluorescence anisotropy was calculated according to the equation [25]:

$$r = (I_{vv} - I_{vh} \cdot G)/(I_{vv} + 2I_{vh} \cdot G)$$

where I_{vv} and I_{vh} are the components of emitted light intensity which are parallel and perpendicular, respectively, with reference to the direction of polarization of the excitation light, and G is the factor ($G = I_{hv}/I_{hh}$) used to correct for unequal transmission in the optical system. Under these conditions the effect of light scattering was negligible.

2.8. The membrane potential measurement

The membrane potential was measured using the optical probe $DiSC_3(5)$ which responds to the membrane potential of the cell according to a widely used method [26,27]. Briefly, erythrocytes were suspended at a hematocrit of 0.2% in buffered saline, containing 10 mM Tris-HCl, pH 7.4 and 150 mM (KCl+NaCl) with K^+ concentrations ranging from 50 mM to 140 mM. To all suspensions of cells the fluorescent dye was added to give a final concentration of 2 μM. After the time required to stabilize the fluorescence intensity of the dye, valinomycin at a concentration of 1 μM was added to the samples. Valinomycin is an ionophore known to induce a marked K^+ permeability through the membranes. The fluorescence intensities of the dye were measured before and after valinomycin addition and plotted as $(I_o - I_{val})/I_o$ versus the corresponding external K^+ concentration. The intersection point of the curve with the abscissa

corresponds to $[K^+]^{np}_{out}$, i.e. the external potassium concentration, for which no change of fluorescence intensity occurs upon the addition of valinomycin. The membrane potential was calculated using the Nernst equation:

$$E_m = (RT/F) \ln [K^+]^{np}_{out}/[K^+]_{in}$$

where E_m is the membrane potential, R is the gas constant, F is the Faraday constant, and $[K^+]_{in}$ is the cellular potassium concentration for erythrocytes. The value of 150 mM was used as the internal K^+ concentration in red blood cells. $DiSC_3(5)$ and valinomycin were added as concentrated ethanolic solutions. The final ethanol concentration in the samples did not exceed 0.4%. Fluorescence was excited at 625 nm and registered at 660 nm with a Perkin-Elmer LS-5B spectrofluorimeter.

2.9. RBC membrane anion exchange protein modification

Inorganic anion transport across the RBC membranes was inhibited by cell modification with the well known erythrocyte band 3 protein blockers DIDS or SITS [28,29]. Cells (hematocrit 10%) in PBS were pretreated with 0.5–1.0 mM of SITS or DIDS at 37°C during 30 min.

2.10. The potassium leakage

The potassium leakage from RBCs was measured as the amount of K^+ ions in the supernatant of the cell suspension treated with nitrite in potassium-free media. The K^+ concentrations were determined by the atomic emission spectroscopy method using a SpectrAA 300/400 photometer (Varian).

3. Results

3.1. Intracellular processes in red blood cells induced by $NaNO_2$

The results reported demonstrate the oxidative processes in RBCs induced by $NaNO_2$. The changes in intracellular metHb and reduced GSH levels in RBC after exposure to $NaNO_2$ are shown in Figs. 1 and 2. The figures reveal a significant increase of

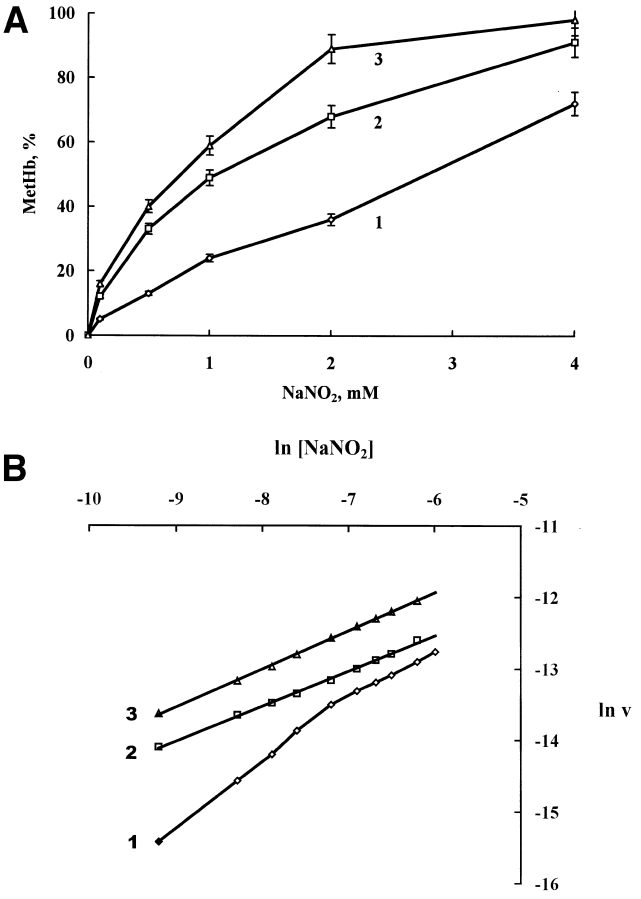

Fig. 1. Oxidative processes in RBCs after exposure to 0–4 mM $NaNO_2$. (A) metHb accumulation in whole cells (1, 2) and in the hemolysates (3). (B) The same results presented according to the equation $\ln v = \ln k + n \cdot \ln c$ (see text). RBCs (or hemolysates) were treated with $NaNO_2$ at 4°C (1) and 37°C (2, 3) in PBS, pH 7.4 (or in 0.005 M phosphate buffer, pH 7.4), for 30 min.

metHb level (Fig. 1) and an initial slight increase in GSH concentration followed by a decrease of its level as a result of oxidation (above 2 mM $NaNO_2$) (Fig. 2, curve 1). The level of reduced GSH in erythrocytes preliminarily oxidized by *t*-BHP increased slightly in the presence of nitrite (Fig. 2, curve 2). The total (reduced GSH and oxidized GSSG) glutathione level did not significantly change under nitrite treatment (Fig. 3, curve 1). The fraction of glutathione bound to proteins in RBC as mixed protein-glutathione disulfides (PSSG) after nitrite treatment increased at above 1 mM oxidant concentration (Fig. 3, curve 2). The rate of oxyHb oxidation in the hemolysates (the initial oxyHb concentrations are the

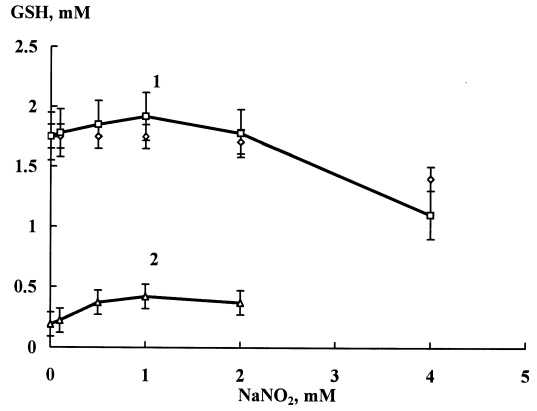

Fig. 2. Oxidative processes in RBCs after exposure to 0–4 mM $NaNO_2$. GSH level in whole cells (1) and in cells pretreated with 0.5 mM *t*-BHP (2). RBCs were treated with $NaNO_2$ in PBS, pH 7.4, 37°C, for 30 min.

same in cell suspensions and hemolysates) was higher in comparison to that of intracellular oxyHb oxidation (Fig. 1). As seen from Figs. 4A and 5 the processes of intracellular oxyHb and GSH oxidations are slow.

The activation energy of the process of intracellular metHb formation under erythrocyte treatment with nitrite, determined from the temperature dependences of the metHb formation rate, is equal to 16.7 ± 1.8 kJ/mol in comparison to 12.8 ± 1.5 kJ/mol for the process of metHb formation in hemolysates.

3.2. Membranous transformations of RBS treated with nitrite ions

We used the potential sensitive anionic fluorescent dye $DiSC_3(5)$ to monitor changes in membrane electrical potential of RBC treated with sodium nitrite. The results reported in Fig. 6 indicate a significantly higher electrical potential of erythrocyte membranes after cell exposure to nitrite. Membrane hyperpolarization was observed at oxidant concentrations higher than 0.25 mM. At the same time even long incuba-

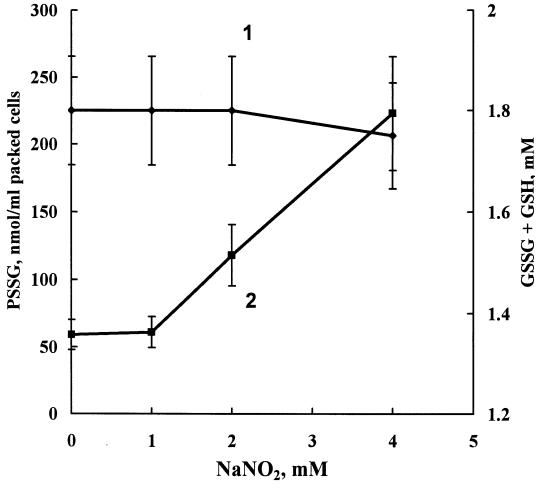

Fig. 3. The influence of $NaNO_2$ (0–4 mM) treatment on the intracellular level of total glutathione (GSSG+GSH) (1) and the level of mixed protein-glutathione disulfides (PSSG) (2). PBS, pH 7.4, 37°C, 30 min of incubation. The concentrations of different forms of glutathione were measured in the equivalents of reduced GSH.

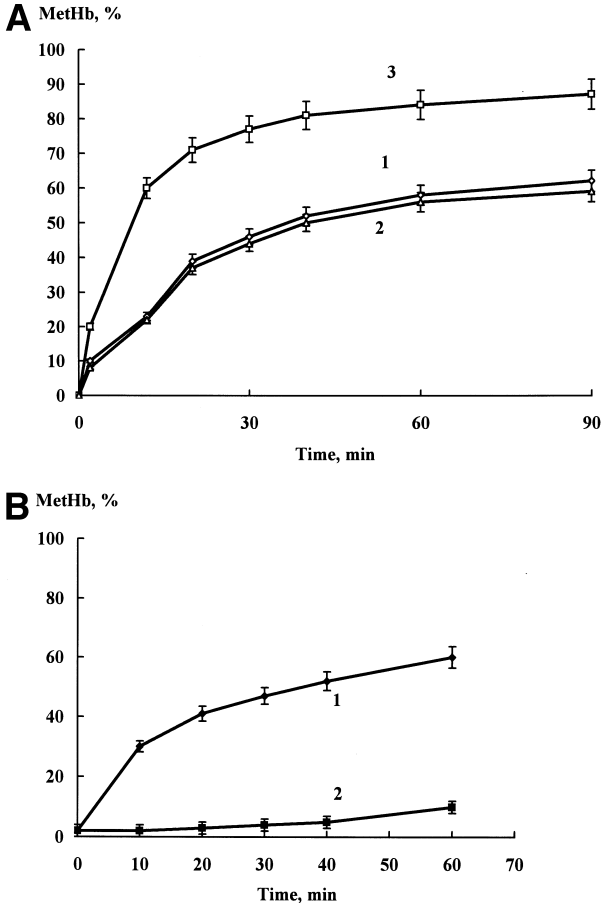

Fig. 4. The time course of intracellular metHb formation (A) after RBCs exposure to 1 mM (1, 2) and 4 mM $NaNO_2$ (3) and (B) after RBC exposure to 4 mM $K_2Cr_2O_7$ (1, 2), PBS, pH 7.4, 37°C. Erythrocytes were treated with oxidative agents before (1, 3) or after (2) preliminary cell modification by 1 mM DIDS.

tion of NO_2^--treated RBC in potassium-free media did not result in K^+ ion leakage from the erythrocytes, as demonstrated by the extracellular potassium measurements, using atomic emission spectroscopy (not shown).

The membrane hyperpolarization was accompanied by a significant decrease in membrane fluidity measured by fluorescence anisotropy of TMA-DPH incorporated into membranes of treated cells (Fig. 6). The osmotic fragility of the erythrocytes treated with $NaNO_2$ slightly increased (data not shown), but we did not observe any hemolysis as a result of nitrite-induced oxidative stress.

The possible contribution of the band 3 protein (anion exchange protein) to the transport of nitrite anion through the cell membrane was studied by examining the effect of blocking band 3 protein function on the rate of intracellular hemoglobin oxidation by externally added nitrite. The rate of intracellular metHb formation under erythrocyte treatment with NO_2^- did not change significantly after preliminary band 3 protein modification by 0.5–1.0 mM SITS or DIDS (Fig. 4A). These two agents are well known inhibitors of band 3 protein function in the erythrocyte membrane [28,29]. At the same time such a modification of the anion carrier protein significantly decreased the rate of intracellular oxyHb oxidation by another anion $Cr_2O_7^{2-}$ known to penetrate via band 3 protein (Fig. 4B).

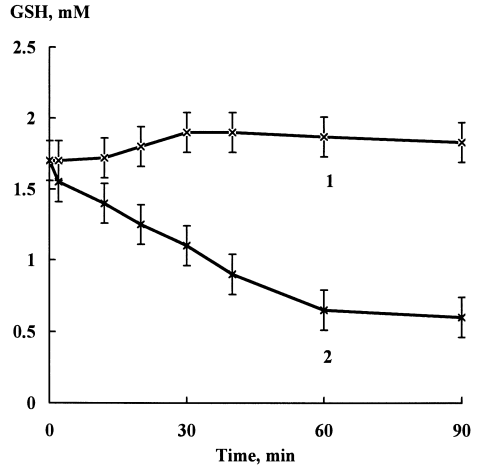

Fig. 5. Time course of intracellular GSH oxidation after RBC exposure to 1 mM (1) and 4 mM (2) $NaNO_2$. Erythrocytes were treated with $NaNO_2$ at 37°C, PBS, pH 7.4.

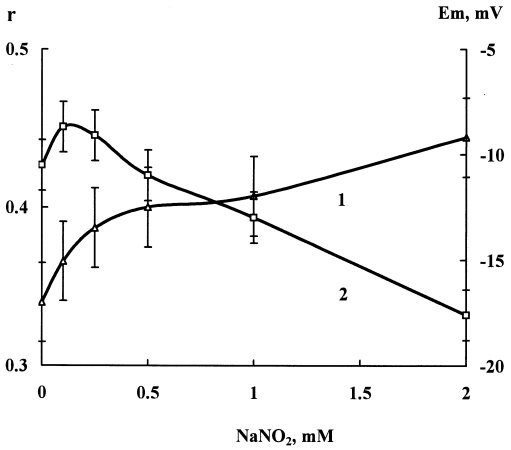

Fig. 6. The dependences of the fluorescence anisotropy r (1) of TMA-DPH incorporated into NO_2^--treated RBCs and membrane potential E_m (2) of these cells on the nitrite concentration. RBCs (10% hematocrit) were treated with different $NaNO_2$ concentrations at 37°C in PBS, pH 7.4, for 30 min.

So, under these conditions the anion transporting protein was really blocked, but the rate of nitrite transport through the membrane did not change.

The change in the erythrocyte membrane potential in the presence of valinomycin (10^{-5} M) also did not significantly influence the rate of intracellular oxyHb oxidation (data not shown).

3.3. pH dependences of oxidative processes in RBC

The rates of nitrite-induced oxidative processes depend largely on the pH of erythrocyte suspension media (Fig. 7A). The rates of metHb formation and GSH oxidation after nitrite treatment were small when the cells were suspended in media with pH higher than 7.5 and significantly increased as pH decreased from 7.5 to 6.5. At pH below 6.5 oxyHb completely converted to the met form at the nitrite concentration used.

In the case of oxidative processes induced by another agent, t-BHP, the rate of oxyHb oxidation increased with decreasing pH from 7.5 to 5.5 (Fig. 7B). The pattern of the dependence of oxyHb oxidation by nitrite on pH is similar to that of oxyHb oxidation by t-BHP in RBC. At the same time the rate of lipid peroxidation (TBARS formation) induced by t-BHP was highest at pH 7.4–7.5 and decreased at both lower and higher pH.

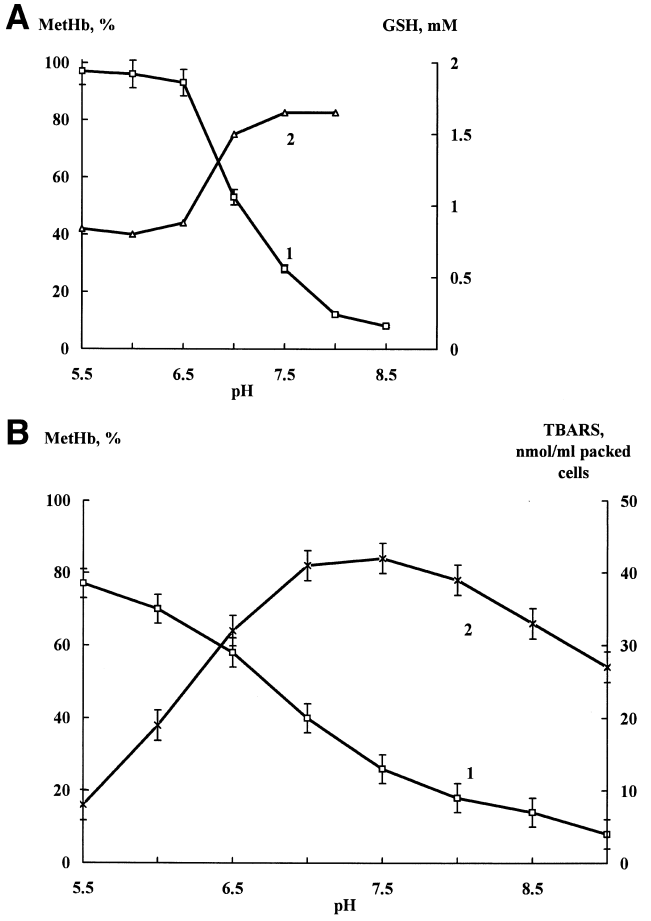

Fig. 7. pH dependences of the rates of (A) metHb formation (1) and GSH oxidation (2) in RBCs treated with 1.5 mM $NaNO_2$ at 22°C for 30 min and (B) metHb formation (1) and TBARS accumulation (2) in RBCs treated with 1 mM *t*-BHP at 22°C for 1 h.

4. Discussion

4.1. Intracellular processes induced by nitrite treatment of RBC

After penetration into erythrocytes nitrite ion interacts with the components of erythrocyte cytoplasm. The reaction of NO_2^- with oxyHb is well known. A complex chain ion-radical reaction mechanism which includes the radical ($^{\bullet}Hb^{4+}$) as a chain propagator has been presented [14–16]. The reaction is characterized by a lag period followed by a rapid, autocatalytic phase. Hydrogen peroxide can be definitely identified as the reaction intermediate. The stoichiometry of the overall oxidation reaction is [14–16]:

$$4HbO_2 + 4NO_2^- + 4H^+ \rightarrow 4Hb^{3+} + 4NO_2^- + O_2 + 2H_2O$$

The nitrite (metHb former) is a one-electron reducing agent. The slow phase of oxyHb oxidation by nitrite is accompanied by the generation of metHb, $NO_2^{\bullet}$, H_2O_2, and, perhaps, $O_2^{\bullet}$ radicals [16,30]:

$$HbO_2 + NO_2^- \rightarrow Hb^{3+} + NO_2^{\bullet} + H_2O_2$$

The reaction of metHb with H_2O_2 resulting in generation of ferrylHb radical ($^{\bullet}Hb^{4+}$) is the basis for the autocatalytic phase [14–16]:

$$Hb^{3+} + H_2O_2 \rightarrow {}^{\bullet}Hb^{4+} + H_2O$$

In this autocatalytic phase $NO_2^{\bullet}$ reacts with a second oxyHb molecule:

$$HbO_2 + NO_2^{\bullet} \rightarrow Hb^{3+} + NO_2^- + O_2$$

as well as with the ferryl form of Hb. Both these reactions lead to metHb formation [16].

Fig. 1A shows the metHb accumulation processes in water solutions (hemolysates) and in erythrocytes after exposure to nitrite. The rates of metHb accumulation in hemolysates and in RBC treated with nitrite are represented as a function of nitrite concentration according to the equation:

$$v = kc^n,$$

where v is the rate of the reaction determined as [metHb]/time of reaction, k is the rate constant, c is the nitrate concentration and n is the order of the reaction with respect to nitrite. (The hemoglobin concentration in cell suspensions and in hemolysates was the constant value in our experiments.) This equation can be transformed

$$\ln v = \ln k + n \times \ln c$$

and the dependences of the rates of metHb accumulation on the nitrite concentration can be plotted in logarithmic coordinates as shown in Fig. 1B. A slope of this dependence represents the order of the reaction.

In agreement with a two-phase mechanism of oxyHb oxidation there are two parts of the concentration dependence for the rate of erythrocyte oxida-

tion at 4°C (as well as for hemoglobin solution): the slow phase of reaction with the order $n = 1.0$, and the rapid, autocatalytic phase with the order $n = 0.5$, where one heme moiety of hemoglobin reacts with 0.5 molecule of nitrite. At higher temperatures (22°C, 37°C) we can observe only the autocatalytic phase of the reaction ($n = 0.5$).

The activation energies of oxidation of the intracellular oxyHb or oxyHb in hemolysates by nitrite are much smaller (16.7 ± 1.8 kJ/mol or 12.8 ± 1.5 kJ/mol, respectively) as compared to that (39.0 ± 5.0 kJ/mol) obtained by us for oxidation of oxyHb in water solution by ferricyanide.

The radicals produced in the reaction of intracellular hemoglobin oxidation converted intracellular GSH to GSSG. For example:

$$GS^- + NO_2^{\bullet} \rightarrow GS^{\bullet} + NO_2^-$$

The glutathione thiyl radicals easily recombine to yield the disulfide form GSSG [31]. We did not observe any significant change of the total glutathione (GSH+GSSG) level in the erythrocytes treated with nitrite (Fig. 3). GSH oxidation in the cell occurred only at high oxidant concentrations (above 2.0 mM $NaNO_2$) in agreement with early observations [32], when a significant part of the intracellular oxyHb was converted to the met form (Figs. 1 and 2). So, oxyHb oxidation is the first step of the intracellular reaction induced by NO_2^- in erythrocytes. Recently it was shown that GSH is not involved in the mechanism of oxyHb oxidation by nitrite in the cytosol of erythrocytes [4]. We did not observe any GSH oxidation by nitrite in aqueous solution.

At transient nitrite concentrations we observed some increase in the cellular GSH level. It may be due to some activation of intracellular enzymes of the pentoso-phosphate pathway and glutathione reductase by nitrite [12]. After preliminary oxidation of erythrocytes by another agent, *t*-BHP, the nitrite treatment of such RBCs increased the level of GSH (Fig. 2). About 10% of total glutathione was bound to erythrocyte proteins (mainly hemoglobin) as mixed protein-glutathione disulfides at high (4 mM) nitrite concentrations (Fig. 3). As we have shown earlier, treatment of erythrocytes with nitrite (1 mg/ml) brought about total oxidation of intracellular glutathione and stimulated export of glutathione disulfide from erythrocytes [33].

The rates of nitrite-induced oxidative processes are significantly pH-dependent (Fig. 7A). It may be due to the pH dependence of the oxyHb reaction with nitrite ion. The protonation of distal histidine in the oxyHb molecule significantly changes the reactivity of the heme moiety of the protein. For example, protonated distal histidine ($pK = 6.5$ in myoglobin) catalyzes the process of hemoprotein autooxidation [34]. With decreasing pH the rates of nitrite-induced oxyHb, as well as glutathione oxidation, increased significantly. This fact shows that the radicals generated in the process of oxyHb oxidation oxidize the glutathione. We cannot exclude the effect of nitrite ion protonation ($pK = 3.4$) and the different reactivity of nitrous acid and nitrite ion. At the same time the nitrite-induced oxidation of oxyHb is a proton-coupled process.

As shown by Duncan et al. the antibacterial activity of nitrite significantly increased with decreasing pH (effect of 'acidified nitrite') [9].

4.2. The comparison of RBC oxidation by nitrite and *t*-BHP

Previously we have studied the oxidation process induced by *t*-BHP in erythrocytes and proposed that this oxidation developed as follows [35]:

GSH oxidation → oxyHb oxidation → TBARS formation

In the case of nitrite the first step of oxidation was metHb formation. OxyHb oxidation preceded changes of the glutathione pool in the erythrocytes.

A membrane-soluble antioxidant, butylated hydroxytoluene, added before oxidant treatment, significantly decreased the level of TBARS formation in the case of *t*-BHP and did not influence oxyHb oxidation in the case of either *t*-BHP or NO_2^-.

The rates of oxidative processes induced by *t*-BHP significantly depended on pH (Fig. 7B). With decreasing pH the rate of metHb formation increased. This might be due to the protonation of the distal histidine residue of Hb. (The pH of erythrocyte cytoplasm was changed when we changed the pH of erythrocyte suspension media.) At the same time the rate of TBARS formation decreased within pH range 7.5–5.5.

4.3. Nitrite damage of the RBC membrane

The interaction of nitrate with the cell drastically modified erythrocyte membranes. As a first step we observed RBC membrane hyperpolarization (Fig. 6). The membrane hyperpolarization after NO_2^- treatment may reflect the increase in specific selective membrane permeability to potassium ions. At the same time we did not observe any significant K^+ leak from the erythrocytes after cell treatment with $NaNO_2$. It has been shown that nitrite-induced methemoglobinemia in trout RBC leads to an inhibition of the red cell sodium/proton exchanger by the R-like hemoglobin conformation [36]. Thus, membrane potential changes after RBC exposure to nitrite may reflect the alterations of the activity of membrane ionic exchangers. Membrane hyperpolarization may also be connected with the changes of membrane surface charge and not with potassium leakage. Earlier it was proposed that nitrite ion as well as other anions, thiocyanate, cyanate, or NH_4^+ can act as the proton carrier across the lipid bilayer and gastric secretory membrane [37]. Nitrite in the protonated form can transport protons through the membrane changing the ion distribution between the inside and outside of the cell. Nitrite anion accumulation in the erythrocytes can also influence (increase) membrane potential. Simultaneously with membrane electrical potential changes we observed membrane rigidization (Fig. 6). The decrease in membrane fluidity may result from the changes of the membrane structural state (intramembrane crosslinks and modifications of membrane proteins) due to the oxidative processes in the membrane.

In our experiments we did not observe any significant changes in the enzymatic parameters (Michaelis-Menten constant, maximal reaction rate) of membrane acetylcholinesterase after RBC treatment with $NaNO_2$ even at high concentrations (4 mM) (data not shown). At the same time the osmotic fragility of RBC after such treatment slightly increased. Such RBC membrane perturbations did not disturb membrane integrity: we did not observe any nitrite-induced hemolysis.

4.4. Membrane transport of nitrite

As calculated from our experiments (Fig. 1B), the rates of metHb accumulation in hemolysates and in RBCs at 0.5 mM of nitrate and 37°C are equal to 2.6 $\mu M\ min^{-1}$ and 2.2 $\mu M\ min^{-1}$, respectively. So, the nitrite transportation through the membrane did not significantly limit the process of intracellular oxyHb oxidation.

Contrary to our results, Tsujita et al., using the method of microspectroscopy of hemoglobin in a single RBC, have shown that in contrast to highly diffusible NO, nitrite ions (NO_2^-) seem to enter RBC very slowly, resulting in a negligible formation of metHb in the presence of 5 mM glucose even during a prolonged incubation period [38].

The well known inhibitors of erythrocyte membrane band 3 protein (SIDS or DIDS) did not change the rate of intracellular oxyHb oxidation in our experiments (Fig. 4A). At the same time such a membrane modification completely inhibited the oxyHb oxidation by another anionic oxidant $Cr_2O_7^{2-}$ (Fig. 4B). We suggest on this basis that band 3 protein (erythrocyte anion transporter) does not play a significant role in the membrane transport of nitrite ions. Earlier it has been shown that band 3 protein plays a minor role in the transport of peroxynitrite into erythrocytes and the permeation of the peroxynitrite ($ONOO^-$) in the anionic form into RBCs may be a minor pathway of erythrocyte membrane transport of the peroxynitrite anion/peroxynitrous acid couple [39].

The various transportation ways for nitrite and nitrate in different types of membranes have been discussed. Nitrate ion (symmetrical anion) was reported to permeate through the apical membrane of a secretory epithelial cell by a chloride channel with a minimum pore diameter of 0.55 nm [40]. Recently, three high affinity transport systems for nitrate and nitrite in *Chlamydomonas reinhardtii* (one specific for nitrite, a second specific for nitrate and another bispecific for these two anions) have been shown [41]. In an early work of Gutknecht and Walter it has been suggested that different anions: thiocyanate (SCN^-), nitrite (NO_2^-), cyanate (CNO^-), diffuse through lipid bilayer membranes as corresponding acids, thus dissipating the proton gradient across the membrane [37].

It is possible that nitrite also penetrates into the RBC membrane in the form of nitrous acid. The pK value of HNO_2 is 3.4 at 18°C in aqueous solution, so

in the physiological range of pH most sodium nitrite is present in the form of the nitrite ion, but in the hydrophobic environment of the membrane the pK value may increase significantly. The nondissociated form of nitrite does not require the specific carrier for transportation through the membrane.

5. Conclusions

Nitrite ion/nitrous acid is a member of an extended family of nitrogen oxides and corresponding acids which are interrelated through a number of chemical reactions. Nitrite-induced intracellular oxidative processes in RBC were compared with its membrane effects. We observed that metHb accumulation and the alteration of the glutathione pool were accompanied by membrane hyperpolarization and rigidization and changes in membrane osmotic fragility. Probably, nitrite penetrates into the erythrocyte in a non-dissociated form of nitrous acid. We have shown that band 3 protein does not participate in the transport of nitrite. The changes in cell electrochemical properties (membrane hyperpolarization) may be due to the change in the activities of membrane ion pumps and/or nitrite-anion accumulation in the cell.

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