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The effect of myeloperoxidase isoforms on biophysical properties of red blood cells

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Abstract

Myeloperoxidase (MPO), an oxidant-producing enzyme, stored in azurophilic granules of neutrophils has been recently shown to influence red blood cell (RBC) deformability leading to abnormalities in blood microcirculation. Native MPO is a homodimer, consisting of two identical protomers (monomeric MPO) connected by a single disulfide bond but in inflammatory foci as a result of disulfide cleavage monomeric MPO (hemi-MPO) can also be produced. This study investigated if two MPO isoforms have distinct effects on biophysical properties of RBCs. We have found that hemi-MPO, as well as the dimeric form, bind to the glycophorins A/B and band three protein on RBC's plasma membrane, that lead to reduced cell resistance to osmotic and acidic hemolysis, reduction in cell elasticity, significant changes in cell volume, morphology, and the conductance of RBC plasma membrane ion channels. Furthermore, we have shown for the first time that both dimeric and hemi-MPO lead to phosphatidylserine (PS) exposure on the outer leaflet of RBC membrane. However, the effects of hemi-MPO on the structural and functional properties of RBCs were lower compared to those of dimeric MPO. These findings suggest that the ability of MPO protein to influence RBC's biophysical properties depends on its conformation (dimeric or monomeric isoform). It is intriguing to speculate that hemi-MPO appearance in blood during inflammation can serve as a regulatory mechanism addressed to reduce abnormalities on RBC response, induced by dimeric MPO.

Keywords Monomeric myeloperoxidase · Dimeric myeloperoxidase · Inflammation · RBC · Phosphatidylserine

Introduction

Myeloperoxidase (MPO) is a cationic protein, which is most abundantly expressed in azurophilic granules of neutrophils (2–5% of the total cellular protein). It is a heme-containing glycosylated oxidoreductase, which in addition to its peroxidase activity, catalyzes the production of (pseudo)hypohalous acids (halogenating activity), mainly hypochlorous (HOCl), hypothiocyanous (HOSCN) and hypobromous (HOBr) acids [1–4]. Being strong oxidants and halogenating agents, hypohalous acids interact with many biologically important molecules: nucleic acids, proteins, lipids, carbohydrates, etc. [5, 6]. Due to this, MPO has a bactericidal function. However, excessive production of reactive halogen-containing compounds (reactive halogen species) can lead to host cell and tissue damage, initiating the development of oxidative/halogenative stress and triggering a number of diseases associated with inflammation [6–8].

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MPO can also regulate the function of immune and non-immune cells via its nonenzymatic effects. MPO binding with the cell surface of platelets [9, 10], neutrophils [11, 12], and erythrocytes [13, 14] are able to activate the processes of intracellular signaling, leading to changes in the structural and functional properties of these cells.

Native MPO, released into the extracellular space as a result of neutrophil degranulation, is a homodimer, consisting of two identical protomers connected by a single disulfide bond, each containing light, heavy chains and heme [15]. Synthesis of dimeric MPO from monomeric ones is carried out at the stage of promyelocyte differentiation into granulocytes, as a result of which a dimeric glycosylated heme-containing MPO is formed [16, 17].

Under in vitro conditions, the monomeric form of MPO, termed hemi-myeloperoxidase (hemi-MPO), can be easily formed by a cleavage of disulfide bond by reduction and alkylation, linking two identical protomers in native MPO [18]. Recently, we have shown that monomeric MPO can be formed in vitro by HOCl-induced disulfide bond oxidation [19]. These results suggest the possibility of hemi-MPO formation in inflammatory foci, where the generation of reactive halogen species is increased and various redox reactions are initiated. Indeed, recently we have shown the presence of hemi-MPO in the plasma of patients with marked inflammation [20]. Under in vivo conditions, the appearance of hemi-MPO is also possible as a result of incomplete processing to the mature enzyme [16, 17].

One of current interest is the question of whether the functional properties of two MPO isoforms are different or similar and whether hemi-MPO, as well as the dimeric form, are able to bind to cell surface and regulate intracellular signaling processes.

Recently, we have shown that hemi-MPO induced cytosolic Ca^{2+}-rise, as well as lysozyme and elastase degranulation in human neutrophils, but these effects were much weaker than observed in the case of dimeric MPO [20]. It should be noted that hemi-MPO has the same as dimeric MPO peroxidase and chlorinating activity and retains its bactericidal ability [16, 17, 21].

In this work, we carried out a comparative analysis of the hemi-MPO (obtained by disulfide bond reduction in dimeric MPO) and dimeric MPO effects on the structural and functional properties of red blood cells (RBCs). We have shown that hemi-MPO, as well as the dimeric form, bind to the glycophorins A/B and band 3 protein on RBC's plasma membrane, that led to changes in transmembrane potential, RBC morphology, reduced RBC deformability and reduced resistance to hemolysis. It was for the first time demonstrated that both dimeric and hemi-MPO induced the exposure of phosphatidylserine (PS) to the outer surface of the RBC membrane. However, all observed effects of hemi-MPO were significantly weaker than in the case of dimeric MPO. According to these data, it is intriguing to speculate that decomposition of native MPO into monomers in vivo may serve as a regulatory mechanism aimed to correct RBC function under inflammatory conditions.

Materials and methods

Chemicals

4-Aminobenzoic acid hydrazide (4-ABH), sodium citrate, 4-chloro-1-naphthol, H_2O_2, HEPES, ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA), phorbol 12-myristate 13-acetate (PMA), ionomycin were purchased from Sigma-Aldrich (St. Louis, USA). Sodium L-aspartate was from Alfa Aesar (Ward Hill, USA). High-affinity rat and rabbit polyclonal antibodies against human MPO were prepared as described previously [22]. HL-60, cell culture medium (RPMI-1640), and fetal calf serum (FCS) were purchased from BioloT Ltd (Saint-Petersburg, Russia).

Isolation of dimeric MPO

The HL-60 cell line (promyelocytic leukemia) was used as a source of dimeric MPO. MPO isolated from HL-60 was identical to MPO isolated from human neutrophils by size exclusion chromatography, SDS-PAGE, Western blotting, N-terminal sequence analysis and have the same peroxidase and chlorinating activities [23]. Cells were cultivated at 37 °C and 100% humidity in RPMI-1640 medium, containing 10% FCS, 2 mM glutamine and 25 mM HEPES buffer (pH 7.4), in roller bottles for suspension culture. Once a week, cells were sedimented by centrifugation at 1500 g, the pellet was resuspended in a minimum volume of fresh medium, and 1/5 of the volume of this cell suspension was transferred to a roller bottle containing fresh medium, while the remaining cells were washed three times with phosphate-buffered saline (PBS, 10 mM Na_2HPO_4/KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4), resuspended in 2 volumes of 100 mM Na-acetate buffer (pH 4.7) and frozen. Dimeric MPO was isolated from the extract of thawed HL-60 cells lysed by ultrasound (44 kHz) and purified by affinity chromatography on heparin-Sepharose, hydrophobic chromatography on phenyl-Sepharose, and gel filtration on Sephacryl S-200 HR [24]. Using this method, it is possible to isolate a homogeneous preparation of dimeric MPO with a high specific activity and a purity index (A_{430}/A_{280}) greater than 0.85.

Preparation of hemi-MPO [25]

The hemi form of MPO was prepared by treating dimeric MPO (145 μM) with 2-mercaptoethanol (1:4 molar ratio of

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MPO to 2-mercaptoethanol) for 30 min at 37 °C in 100 mM Na-carbonate buffer, pH 9.4, as described elsewhere [18, 25]. SH-groups were then blocked with iodoacetamide for 30 min at 4 °C (1:20 molar ratio of MPO to iodoacetamide). The resulting protein solution was concentrated in VivaSpin 20 ultrafiltration units (Sartorius, Germany) with a molecular weight cut-off of 30 kDa, with the buffer being exchanged for 100 mM Na-acetate buffer (pH 5.5). Traces of dimeric MPO were separated from hemi-MPO by gel filtration on a Sephacryl S-200 HR column (114 × 1.5 cm) equilibrated with 100 mM Na-acetate buffer (pH 5.5). SDS-PAGE in non-reducing conditions showed a complete absence of the dimeric form in hemi-MPO preparation. It was shown that there were no differences in peroxidase, chlorinating and bactericidal activity between hemi-MPO and dimeric MPO [25]. Concentration of dimeric and hemi-MPO was determined spectrophotometrically using an extinction coefficient of 112,000 $M^{-1} \cdot cm^{-1}$ per heme of MPO.

Isolation of RBCs

Washed RBCs were obtained after two centrifugation cycles at 400 g for 5 min of capillary blood (100 µl) in 10 ml of PBS or venous blood collected in tubes containing 3.8% (w/v) sodium citrate as anticoagulant at a ratio of 9:1 and stored in PBS, containing 10 mM D-glucose at 4 °C. Washed RBCs from capillary blood (1% hematocrit, unless otherwise indicated) were used for AFM, hemolysis, patch-clamp and flow cytometry assays whereas washed RBC from venous blood were used to prepare RBC ghosts (RBCGs) by hypoosmotic hemolysis. Venous blood samples were obtained from healthy donors at Federal State Budgetary Scientific Institution "Institute of Experimental Medicine". All blood donors were volunteers and gave informed consent.

RBCGs preparation

Washed RBCs were mixed with cold hemolysis buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.6, 4 °C) at a 1:20 ratio by volume and incubated at 4 °C for 5 min. Then, the sample was centrifuged twice at 30,000×*g* (30 min, 4 °C) and the RBCG pellet was resuspended with cold hemolysis buffer: by 10 volumes (first centrifugation) and by 3 volumes (second centrifugation). The final RBCG suspension was used for downstream procedures.

Detection of MPO-binding proteins using ligand western blot assay

RBCGs were lysed in SDS-Tris sample buffer (125 mM Tris–HCl, pH 6.8, 2% SDS, 0.1% 2-mercaptoethanol, 0.001% bromphenol blue, and 50% glycerol) at a ratio 1:5 by volume, and 100 µg of total protein was loaded per well of polyacrylamide gel [26]. Using a semi-dry method [27] the separated proteins were transferred on nitrocellulose membranes and the blocking procedure was performed using a blocking solution BSA-T (1% BSA amd 0.05% Tween 20 in PBS). To detect RBC proteins, which bind with MPO isoforms, the membranes were incubated for 30 min with hemi- or dimeric MPO in BSA-T solution, followed by exposure for 1 h to HRP-labeled rabbit anti-human MPO antibody. Each step was accompanied by washing of the membranes three times with BSA-T solution for at least 5 min per washing step. The peroxidase activity was visualized using 4-chloro-1-naphthol plus H_2O_2 system. In the absence of HRP-labeled antibody, basal MPO peroxidase activity was not manifested. There were no difference between MPO and hemi-MPO in binding to the HRP-labeled antibody against MPO as was shown in control dot-blotting experiments. The identity of MPO-binding protein bands on SDS-PAGE gels was confirmed by mass spectrometry after in situ tryptic digestion [28].

Hemolysis detection

A suspension of washed RBCs (30 µl) treated or not with monomeric/dimeric MPO was added to 60 mM NaCl solution (300 µl) to induce hypotonic hemolysis or to phosphate-citrate buffer containing 155 mM NaCl and 4.1 mM Na_2HPO_4/7.9 mM citric acid (300 µl) to induce acidic hemolysis. The process of hemolysis was recorded as changes in light transmission at 670 nm and 37 °C of constantly stirred cell suspensions using analyzer AP2110 (SOLAR, Minsk, Belarus). To quantify the hemolysis process the following parameters were used: *G*, maximal extent of hemolysis, i.e., the maximal level of light transmission of cell suspension at the plateau, and t_{50}, the time point when the change in light transmission has reached its half-maximal value.

Atomic force microscopy (AFM) measurements

RBCs were treated with monomeric/dimeric MPO for 10 min at room temperature and then fixed in 1.5% glutaraldehyde for 30 min. Fixed RBCs were washed by four-step centrifugation at 400 g for 3 min and the RBC pellet was resuspended twice in PBS and twice in distilled water. Washed RBCs were placed on a glass slide and air-dried for several hours. All steps were performed at room temperature.

The images of RBC's surface membrane were obtained using a NT-206 microscope (MicroTestMachines, Minsk, Belarus) working in the contact mode using the software of the microscope. Standart cantilevers NSC 11A («Mikro-Masch» Co, Estonia) with a spring constant of 3 N/m were used. Tip radii were checked by using a standard TGT01 silicon grating from NT-MDT (Moscow, Russia) and were

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10 nm for topography visualization and 60 nm for cell stiffness determination. Surface profiles were obtained using scan sizes of 14 × 14 mm at a scan rate of 3 Hz. The resulting image (topographic image) was recorded as a surface height distribution $Z(X, Y)$. For each scanned cell, the height H (maximum cell height), the concave depth h (minimum height of the cell), the diameter of RBC – d and the relative concave depth – k were determined: $k = (H–h)/h \cdot 100\%$.

The force spectroscopy regime was used to determine local elastic properties of RBCs. At least three force curves from the peripheral part of the randomly selected cells (7–10 cells) for each treatment were recorded. The cell Young's modulus was calculated as described earlier [29] using Hertz model and used as a measure of RBC stiffness. The indentation depth was 15 nm to avoid the influence of a rigid substrate on the magnitude of the estimating Young's modulus [30].

Light microscopy

To observe changes in RBC morphology, induced by MPO isoforms, RBCs were suspended in PBS, pH 7.4, with 1 mM $CaCl_2$, placed in a Petri dish and transferred to an optical microscope for analysis. The transmitted light images of the RBCs were recorded before (control) and after MPO addition to cell suspension at time intervals of 15–60 s for 15 min using an optical microscope Olympus BX51WI (Tokyo, Japan), LUMPlan objective (40 ×/0.80) and digital camera OSCAR 45 (Taiwan). Quantitative analysis was performed using the analyzer Meco-Hemo (Mecos, Russia) counting approximately 500 cells per each image.

Measurement of RBC membrane potential by patch-clamp technique

Washed RBCs (5 µl) were carefully placed in the bottom of a Petri dish, filled with 5 ml of external buffer solution (145 mM NaCl, 10 mM HEPES, 10 mM D-glucose, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.4, osmolarity 290 mOsm). Patch pipettes with tip resistance 10–20 MΩ were prepared from borosilicate glass before each experiment using a puller Sutter P-97 (HEKA Elektronik, GmbH) and filled with internal buffer solution (5 mM NaCl, 10 mM HEPES, 145 mM KCl, 1 mM $MgCl_2$, 0.3 mM $CaCl_2$, 3 mM EGTA, pH 7.2, osmolarity 280 mOsm). A micromanipulator MP-225 (Sutter Instrument) was used to bring the patch pipette close to a single RBC and then a small negative pressure was applied to the pipette, leading to giga-seal formation (3–10 GΩ). Patch-clamp recordings of membrane potential were carried out in cell-attach configuration in current-clamp mode using an amplifier HEKA EPC 8 (HEKA Elektronik, GmbH), filtered at 1 kHz. When the successful cell-attached configuration was achieved and membrane potential reached the constant values (15–20 mV), dimeric or hemi-MPO was added to bath solution and changes in membrane potential were recorded.

Flow cytometry

To probe PS exposure, washed RBCs were suspended at 0.015% hematocrit in PBS, pH 7.4, with 2 mM $CaCl_2$, treated with monomeric/dimeric MPO or ionomycin/PMA for 15 min at room temperature, stained with Annexin V-Alexa Fluor 647 (100 µg/ml) under protection of light for 5 min at room temperature und used immediately for flow cytometry assay. PS exposure was measured in the FL-6 channel (660 nm) excited at 638 nm. 10,000 cells were measured per each sample.

To measure intracellular Ca^{2+}, RBCs were incubated with 3.5 µM Fluor-3/AM in PBS for 60 min at 37 °C in the dark, followed by centrifugation (300 g, 7 min) and subsequent washing in PBS three times. Fluor-3-loaded RBCs were exposed to 100 nM of hemi-MPO or 100 nM of dimeric MPO or 1 µM of ionomycin (as a positive control) and the aliquots were sampled every minute to detect changes in Fluor-3 fluorescence (525 nm) excited at 488 nm.

Both flow cytometric assays were performed on a Navios (Beckman Coulter, USA) system.

Statistical analysis

Data are expressed as mean ± SD or mean ± SEM, as indicated in the captions to the figures and tables. To analyze differences between mean values of the two groups, the Student t test was used. Differences between mean values of more than two groups were analyzed by ANOVA followed by Student–Newman–Keuls test. Statistical analysis was performed using Origin 7.0 (Northampton, USA) or Statistica software. A p value < 0.05 was considered to be significant.

Results

Interaction of RBCG proteins with hemi-MPO

Recently, it was shown that binding of dimeric MPO to RBC surface is based mostly on electrostatic interactions with the participation of sialic acids and its main targets are band 3 protein (B3) and glycophorin A and B [13, 31]. To check if hemi-MPO binds to the same targets on RBC surface, RBCG proteins were separated by SDS-PAGE (Fig. 1, panel 1) and transferred to a nitrocellulose membrane. Their interaction with hemi-MPO and dimeric MPO were analyzed using ligand Western blotting, using rabbit anti-MPO antibodies labeled with horseradish peroxidase. Rabbit antibodies against MPO did not react with RBCG proteins without

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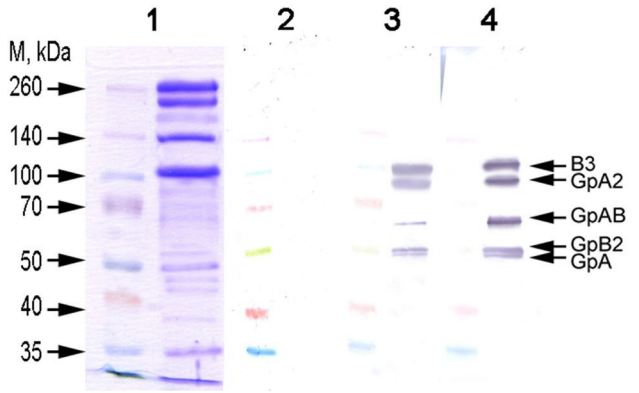

Fig. 1 Binding of human MPO to RBCG proteins. Prestained protein marker (the left lanes in each panel) and RBCG proteins (100 µg per line) separated by SDS-PAGE and transferred on nitrocellulose membranes: panel 1—Coomassie brilliant blue R-250 staining; panel 2, 3 and 4—ligand Western blot staining using: no MPO (panel 2), 200 nM dimeric MPO (panel 3) and 200 nM hemi-MPO (panel 4) and horseradish peroxidase (HRP)-labeled rabbit anti-human MPO antibody (10 µg/ml) of membranes. Arrows represent protein standards (on the left) or positions of specific RBCG proteins and their dimers (on the right)

preliminary addition of MPO (Fig. 1, panel 2). The membrane showed that five dimeric MPO-binding regions were revealed using ligand Western blot assay (Fig. 1, panel 3) corresponding to the band 3 protein (B3) and glycophorin A and B (GpA2, GpAB, GpB2, GpA). These glycoproteins were identified earlier with help of periodic acid-Schiff reagent and by mass-spectrometry [13]. Similar patterns of hemi-MPO binding to the five protein areas were detected (Fig. 1, panel 4). These results indicate that hemi-MPO as well as the dimeric MPO binds to the band 3 protein and glycophorin A and B of the RBC plasma membrane.

To be sure that hemi- and dimeric MPO stably bind to RBC surface proteins in their native environment, we incubated washed RBCs with MPO isoforms for 15 min and then measured MPO concentration in cell supernatants as described earlier [32]. The decrease of dimeric MPO as well as hemi-MPO content in cell supernatant (Supplementary Materials, Fig.S1) indicates that both isoforms stably bind with RBCs.

Effect of hemi-MPO on the RBC elastic properties and their resistance to hemolysis

Hemolysis was initiated by reducing the ionic strength of the medium (osmotic hemolysis) or pH (acidic hemolysis). As shown in Fig. 2a, b hemi-MPO as well as dimeric MPO augmented acidic and osmotic hemolysis in a dose-dependent manner. Thus, the degree of osmotic hemolysis (Fig. 2c) increased, and the half-time of acidic hemolysis decreased (Fig. 2d) for RBCs treated with both MPO forms in comparison to control, indicating a decrease in cell resistance to hemolysis. However, the effect of hemi-MPO was lower in comparison with native dimeric MPO (Fig. 2c, d). It should be noted, that unrelated to MPO positively charged protein human lactoferrin (hLF) with molecular mass 76 kDa similar to that of hemi-MPO did not affect acidic and osmotic hemolysis (data not shown) indicating the specificity of MPO isoforms' effect on RBC resistant to hemolysis.

As MPO can induce the production of hypohalous acids, which are known to initiate RBC hemolysis [33, 34], we next examined the observed effects in the presence of MPO enzymatic activity inhibitor – 4-ABH. As shown in Fig. 2e 4-ABH (50 µM) failed to abrogate hemi-MPO-mediated increase in hemolysis. Furthermore, under hypotonic and acidic conditions (used in present study) MPO peroxidase activity decreased by at least 97%. ABH (50 µM) almost completely suppressed the rest of MPO enzymatic activity (data not shown).

Differences in the hemi-MPO and dimeric MPO effects on RBC mechanical properties were also shown by AFM. To assess the RBC surface elastic properties and cell stiffness, we determined the local Young's modulus for intact RBCs and RBCs treated with both MPO isoforms (Fig. 2f). Hemi-MPO and dimeric MPO caused increase of Young's modulus values by approximately 10% and 30%, respectively (Fig. 2f). These data indicate that both MPO isoforms lead to RBC membrane stabilization and increase in their mechanical stiffness but to a various stage.

Thus, it can be concluded, that binding of hemi-MPO, as well as native MPO with RBC plasma membrane, initiates similar changes in cell structural and functional properties. These hemi-MPO effects do not depend on the catalytic activity of the enzyme and are rather weaker than in the case of dimeric MPO.

Effect of hemi-MPO on size and morphology of RBCs

We have recently shown that RBC treatment with native dimeric MPO led to their volume increase, as evidenced by a marked increase in the number of stomatocytes and microspherocytes [13]. Moreover, the maximum change in cell morphology occurred within the first two min and then the cells reverted back to the morphology of normocytes. In this work, we examined the effect of hemi-MPO on cell morphology and compared it with the effect of dimeric MPO.

During the period of observation (15 min) the morphology of control (untreated) RBCs did not change over time. As expected dimeric MPO addition to RBCs suspension induced cell swelling during the first 15 s as was evidenced by appearance of significant amounts of stomatocytes (Fig. 3e), reduction in echinocyte number (Fig. 3b) and after 15 min of observation led to significant rise in the number

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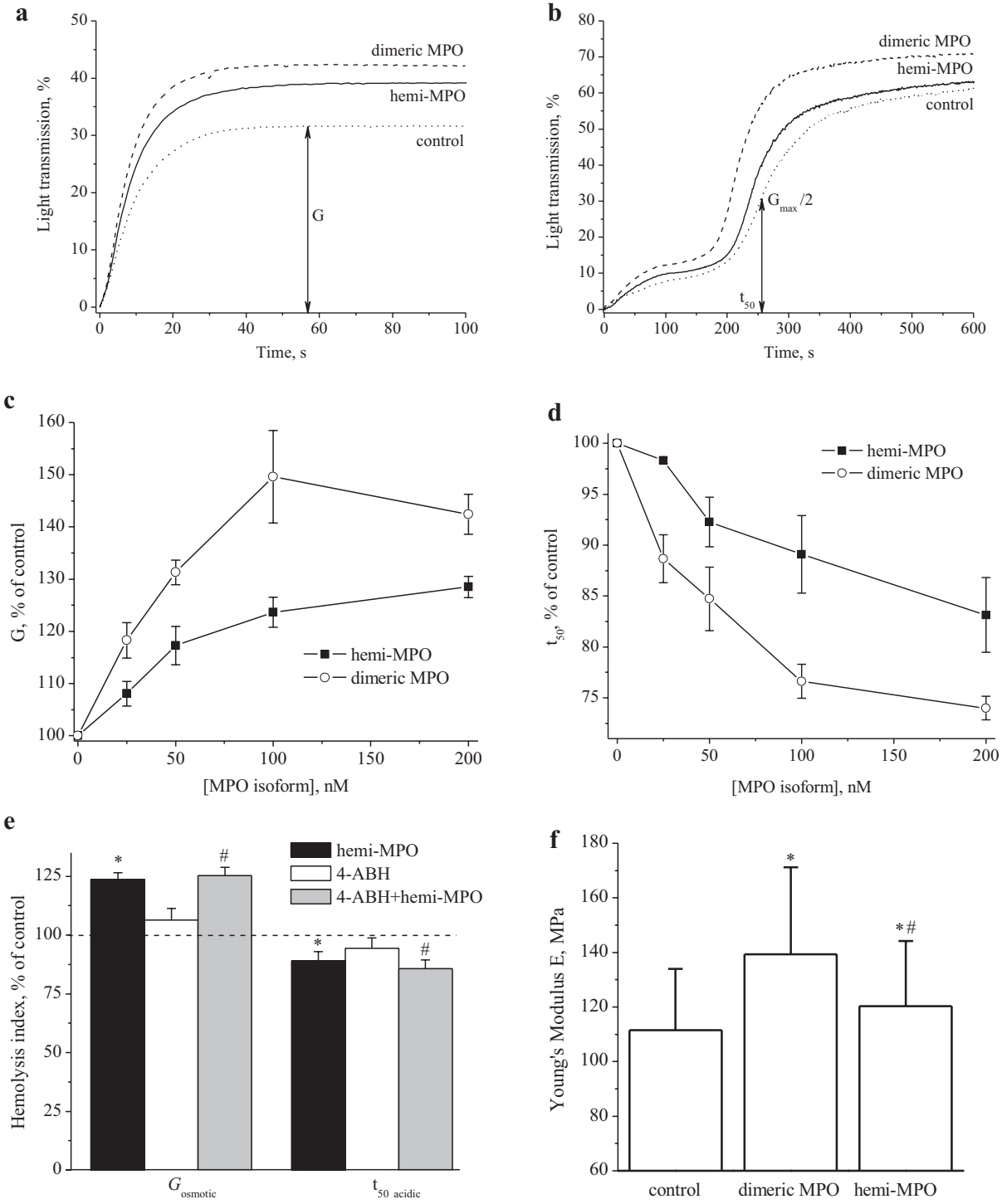

Fig. 2 Effects of MPO isoforms on hemolysis and RBC membrane elasticity. **a, b** Typical kinetics of osmotic (60 mM NaCl) (**a**) and acidic (4.1 mM Na_2HPO_4/7.9 mM citric acid, 155 mM NaCl; pH 2.9) (**b**) RBC hemolysis in the absence (control) and presence of dimeric and hemi-MPO (100 nM). **c, d** The degree (G) and the half-time (t_{50}) of osmotic (**c**) and acidic (**d**) hemolysis in the presence of different concentration dimeric and hemi-MPO. **e** The effect of specific inhibitor of MPO enzymatic activity 4-ABH (50 μM) on the degree of osmotic ($G_{osmotic}$) and the half-time of acidic ($t_{50\ acidic}$) RBC hemolysis in the presence of hemi-MPO (100 nM). The hemolysis index of control MPO untreated RBCs was 100%. **f** The influence of MPO isoforms (25 nM) on the Young's modulus of RBCs. The data are presented as mean ± SD [$n = 3$–5 (**a**–**e**) or $n = 23$–26 (**f**)].*$p < 0.05$ compared to control, #$p < 0.05$ compared to the effect of 4-ABH (**e**) or dimeric MPO (**f**)

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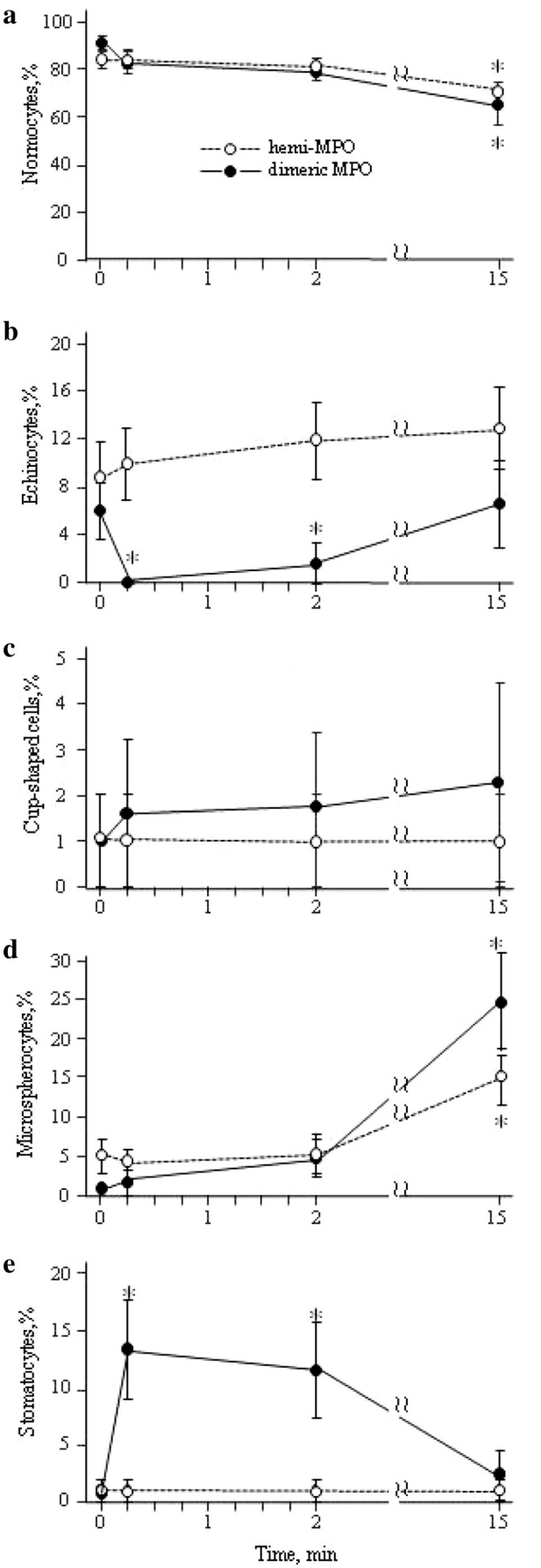

Fig. 3 Changes in RBC morphology after incubating the cells with dimeric MPO (100 nM) or hemi-MPO (100 nM). The number (in %) of normocytes (**a**), echinocytes (**b**), cup-shaped cells (**c**), microspherocytes (**d**) and stomatocytes (**e**) was calculated for 15 s, 2 min, and 15 min after MPO addition. The data are presented as mean ± SEM ($n = 500–550$). *$p < 0.05$ comparing means to untreated control

of microspherocytes (Fig. 3d). Although hemi-MPO did not induce significant changes in the number of stomatocytes and echinocytes (Fig. 3e, b), the final increase in the number of microspherocytes was significant (Fig. 3d), however, this effect was less pronounced than in the case of dimeric MPO.

It should be noted that the observed appearance of microspherocytes in cell suspension indicates about the MPO-induced increase in cell volume. Indeed, changes in RBC volume, induced by both MPO isoforms, were observed by AFM (Fig. 4, Table 1). It was shown that RBCs treatment with dimeric MPO led to a decrease in concave depth, as evidenced by a significant change in the parameters *h* and *k*, while other linear cell sizes (height, *H* and diameter, *d*) were unaffected (Table 1, Fig. 4c). In the presence of hemi-MPO, a decrease of the relative concave depth (*k*) was also observed, however, this change was lower, compared to native dimeric MPO (Fig. 4).

Thus, the obtained results indicate that hemi-MPO, similarly to the dimeric isoform of the enzyme, induces changes in RBC morphology and increase in their volume, but to a much lesser extent than dimeric MPO.

Hemi-MPO effect on RBC membrane potential

Changes in morphology and RBC volume are closely linked to ionic conductivity of plasma membrane. Thus, recently, we have shown that MPO-induced increase in RBC volume is associated with depolarization of plasma membrane, while the subsequent restoration of cell morphology and volume—with plasma membrane hyperpolarization [13]. In the present work, we also examined whether hemi-MPO had an influence on RBC membrane potential. Using a "cell-attach" patch clamp technique, it was shown, that like in the case with dimeric MPO, the addition of hemi-MPO to RBC suspension induced a two-stage change in membrane potential: fast membrane depolarization, followed by a prolonged hyperpolarization (more than 10 min) (Fig. 5a). As expected, the effect of hemi-MPO at both stages: depolarization and hyperpolarization were lower compared to dimeric isoform of MPO (Fig. 5a, b).

It should be noted, that all described changes in structural and functional properties of RBCs, induced by both MPO isoforms, were observed only in the medium containing Ca^{2+} ions. No apparent changes in morphology, cell sizes or ion permeability occurred in calcium-free medium (data not shown). Actually, we have shown previously [13], that

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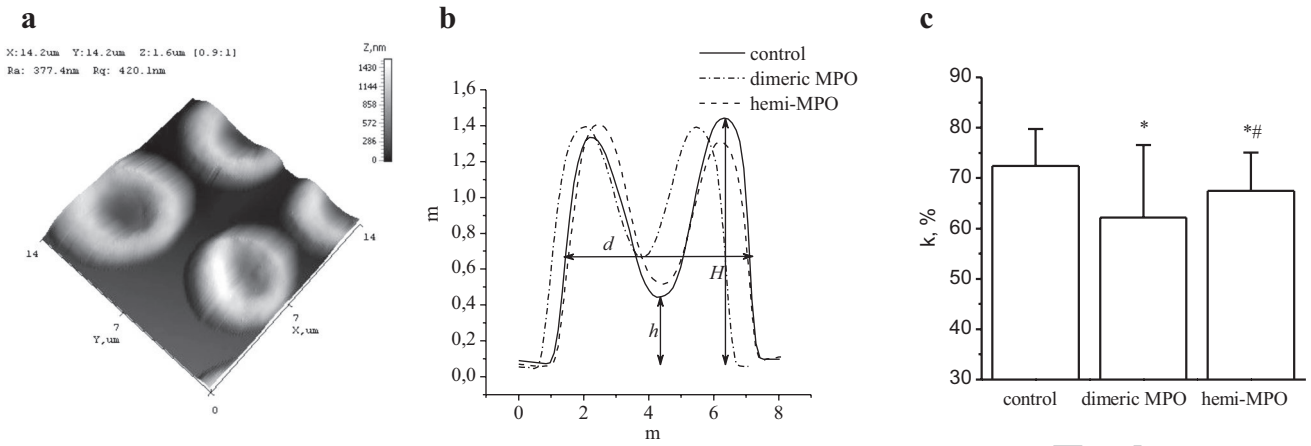

Fig. 4 AFM-images and surface topography of RBCs. **a** AFM 3D-image of RBCs fixed with 1.5% glutaraldehyde for 30 min. **b** The profiles of the RBC treated with dimeric MPO (100 nM), hemi-MPO (100 nM), or the vehicle (control), used to calculate cell diameter – *d* maximal cell height (*H*), concave height (*h*), and relative concave depth, calculated as: $k=(H–h)/h\cdot100\%$. **c** Changes in the relative concave depth of the RBC (*k*) after 2 min incubation with dimeric or hemi-MPO (100 nM). The data are presented as mean ± SD ($n=52–58$). $*p<0.05$ comparing means to untreated control, $\#p<0.05$ comparing means to the effect of dimeric MPO

Table 1 Changes in RBC size in the presence of dimeric and hemi-MPO (100 nM)

	H, μm	*h*, μm	*d*, μm
Control	1.36 ± 0.15	0.40 ± 0.15	5.64 ± 0.29
Dimeric MPO	1.36 ± 0.14	0.51 ± 0.43*	5.45 ± 0.36
Hemi-MPO	1.35 ± 0.15	0.42 ± 0.08#	5.46 ± 0.46

H RBC height (maximum height of the cell), *h* RBC concave height (minimum height of the cell), *d* RBC diameter. The data are presented as mean ± SD ($n=52–58$) $*p<0.05$ compared with control, $\#p<0.05$ compared with the effect of dimeric MPO

binding of native MPO to RBC plasma membrane induces Ca^{2+} entry into the cytosol of cells. In present work, hemi-MPO was also capable to induce rise in cytosolic Ca^{2+} concentration as measured by flow cytometry in Fluor-3 loaded RBCs (Fig. 6) but this effect was lower compared to the Ca^{2+}-response induced by dimeric MPO and Ca^{2+}-ionophore ionomycine.

Since intracellular Ca^{2+}-rise can activate phospholipid scramblase, that bidirectionally and nonspecifically transports phospholipids, leading to PS exposure on cell external

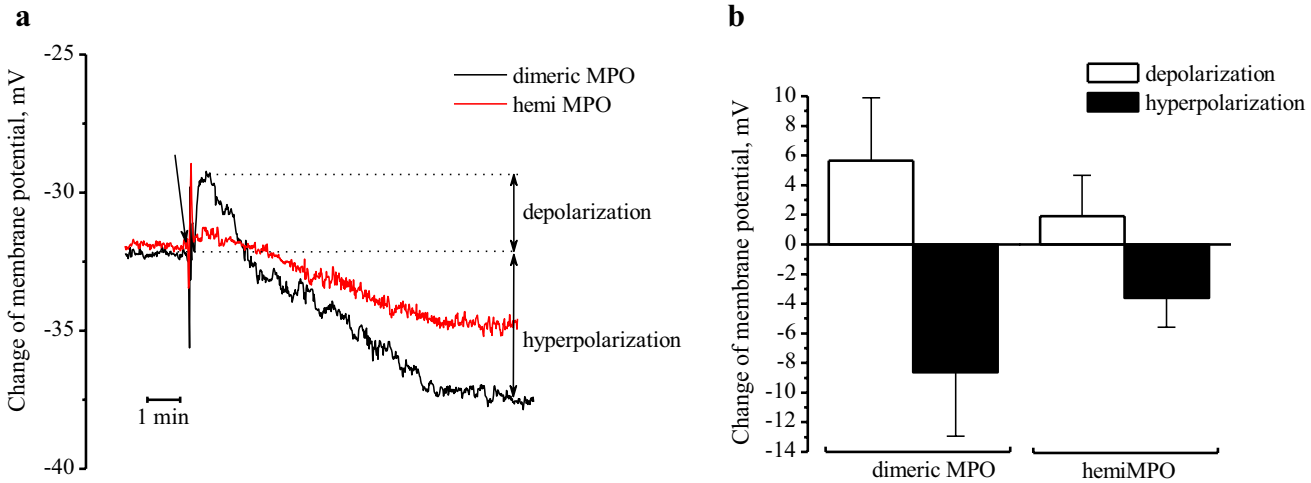

Fig. 5 Effect of MPO isoforms on RBC membrane potential. **a** The kinetics of membrane potential induced by dimeric or hemi-MPO (100 nM), which show depolarization and hyperpolarization phases. **b** Values of depolarization and hyperpolarization effects of MPO isoforms on RBC membrane potential. Arrow indicates the moment of MPO isoform addition. The data are presented as mean ± SD ($n=7–8$)

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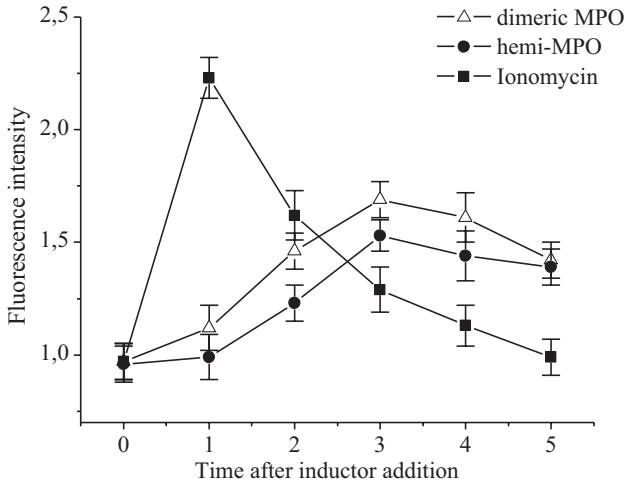

Fig. 6 Effect of MPO isoforms on cytosolic Ca^{2+} concentration. Changes in mean fluorescence intensity of Fluor-3 loaded RBCs, treated with dimeric MPO (100 nM), hemi-MPO (100 nM) or ionomycin (1 μM), detected by flow cytometry assay. The data are presented as mean ± SD ($n = 3–5$)

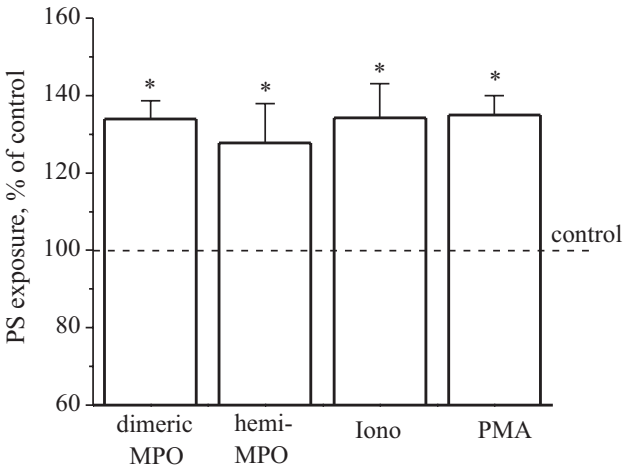

Fig. 7 Effect of MPO isoforms on PS exposure on the outer leaflet of RBCs. Prior annexin V-Alexa Fluor 647 staining RBCs were pretreated with dimeric MPO (100 nM), hemi-MPO (100 nM), PMA (5 μM), ionomycin (1 μM) or the vehicle (control) for 15 min at room temperature in PBS, containing 2 mM $CaCl_2$. PS exposure (Annexin V binding) was determined by change in mean fluorescence intensity, % of control. The data are presented as mean ± SD ($n = 3–4$). *$p < 0.05$ comparing means to untreated control

leaflet [35] and considering recent data, that PS exposure is controlled by membrane hyperpolarization due to Ca^{2+}-dependent Gardos channel opening [36], it was intriguing to investigate if native dimeric and hemi-MPO lead to PS exposure on the RBC's membrane.

PS exposure in RBCs, treated with dimeric and hemi-MPO

To determine if MPO isoforms are able to induce PS exposure on the outer RBC's leaflet, cells were preincubated with native dimeric or hemi-MPO for 15 min and stained with annexin V for PS detection by flow cytometry. As a positive control, we used calcium ionophore ionomycin (1 μM) and PKC activator PMA (5 μM), which were shown to induce PS exposure in RBCs [36–38]. As shown on Fig. 7 RBC treatment with both dimeric MPO and hemi-MPO led to a significant increase in PS exposure by 34% and 22%, respectively. The effect of dimeric MPO was comparable to that of ionomycin and PMA. However, according to the previous results, the effect of hemi-MPO was less pronounced.

Discussion

Today, along with wide investigation of MPO enzymatic activity, great attention is paid to its ability to bind to plasma membrane of blood cells and regulate their structural and functional properties. This ability doesn't depend on the catalytic activity of the enzyme, but is largely due to the peculiarities of the structure of the MPO molecule. In this work, we have shown, that the decomposition of dimeric MPO into monomers is accompanied by a decrease in its ability to regulate the structural and functional properties of red blood cells.

The peculiarity of MPO structure is that mature MPO, which is stored in azurophilic granules of fully differentiated neutrophils, is a dimer (~ 145 kDa), consisting of identical heme-containing protomers connected by a disulfide bond. Native dimeric MPO is able to bind to the plasma membrane and regulate the functional responses of various cells.

Thus, binding of native MPO to CD11b/CD18, a major neutrophil adhesion receptor, leads to tyrosine phosphorylation of a number of proteins and as a result stimulates degranulation [12], adhesion, and also increases the survival of these cells [39]. However, as has been shown previously [20], abnormal MPO conformation is accompanied by a decrease in its ability to regulate the functional activity of neutrophils. The reductive alkylation of MPO leads to its inability to enhance neutrophil adhesion [40]. Recently, we have shown that hemi-MPO, as well as MPO modified by hypochlorous acid (MPO-HOCl), lost its ability to prime NADPH-oxidase of neutrophils [20]. In addition, it was found that hemi-MPO to a much lesser extent than dimeric MPO-stimulated rise in cytosolic calcium and lysozyme exocytosis in neutrophils, and the capacity of monomeric MPO to delay apoptosis of neutrophils and increase their lifespan was weaker than that of dimeric MPO [20].

Previous studies with RBCs demonstrated that MPO-HOCl, in contrast to native dimeric MPO, lost its ability

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to bind to plasma membrane of RBC and regulate their structural and functional properties [13]. Apparently, this effect was due to a decrease in the net positive charge of the MPO molecule, resulted from halogenation of its amino groups by HOCl, that led to a decrease in the electrostatic interaction with negatively charged RBC plasma membrane proteins. In present study, we have shown for the first time that in contrast to MPO-HOCl [13], hemi-MPO, obtained from native MPO by disulfide cleavage, retained the ability of the enzyme to bind to RBC surface (Fig. 1). Since dimeric MPO dissociation into two hemi-MPO molecules due to disulfide bond reduction preserves the charge of the hemi-MPO molecules, then, apparently, the electrostatic interaction of hemi-MPO with RBC proteins is conserved.

Binding of hemi-MPO, as well as binding of dimeric MPO with RBC's membrane proteins, reduced cell resistance to osmotic and acidic hemolysis as well as cell elasticity (Fig. 2), led to significant changes in cell volume, morphology (Table 1, Figs. 3, 4), the conductance of plasma membrane ion channels (Fig. 5) and cytosolic Ca^{2+} concentration of RBCs (Fig. 6). It has been shown for the first time that both dimeric and hemi-MPO contribute to the formation of PS-positive RBCs (Fig. 7). These results are of great importance, as the exposure of PS on the outer membrane leaflet of RBCs serves as a signal for eryptosis, a mechanism for the RBC clearance from blood circulation and also lead to adhesion of RBCs to endothelium in some diseases such as sickle cell anemia, malaria, and diabetes [41].

However, the effects of hemi-MPO on the structural and functional properties of RBCs were lower compared with those of dimeric MPO. The possible reason is the presence of two receptor-binding sites on native dimeric MPO molecule in contrast to one binding site for hemi-MPO. Dimeric MPO, being a bivalent ligand, when binds to its corresponding receptors, can lead to their clustering that may have a significant effect on intracellular signaling [42, 43]. On the other hand, it was shown that MPO-binding proteins on RBC membrane: band 3 protein and glycophorin A, form a complex [44, 45]. Furthermore, as bivalent ligands may possess higher binding affinity to clustered receptors compared to monovalent ligands [42, 43], dimeric MPO effect on the structural and functional RBC properties may be more pronounced compared to hemi-MPO.

Thus, the ability of MPO protein to influence RBC's biophysical properties depends on its conformation (dimeric or monomeric isoform). It is intriguing to speculate that hemi-MPO appearance in blood during inflammation, as it was shown earlier [20], can serve as a regulatory mechanism addressed to reduce abnormalities on RBC response.

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Compliance with ethical standards

Conflict of interest The authors declare no competing financial and nonfinancial interests.

Ethical approval This work was approved by the protocol of the Local Ethics Committee at Federal State Budgetary Scientific Institution "Institute of Experimental Medicine".

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