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Modulation of RBC volume distributions by oxidants (phenazine methosulfate and tert-butyl hydroperoxide): Role of Gardos channel activation

Irina L. Lisovskaya *, Irina M. Shcherbachenko, Rimma I. Volkova, Vladimir P. Tikhonov

National Scientific Centre for Hematology, Novyi Zykovsky pr. 4a, Moscow 125167, Russia

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ABSTRACT

A study was made comparing the effects of two oxidants – phenazine methosulfate (50–1500 μM)+10 mM ascorbate and t-butyl hydroperoxide (1–3 mM) – on the volume-related parameters of normal human red blood cells. Incubation with either oxidative system for 20–30 min resulted in red blood cell density and osmotic resistance distribution shifts. Treatment with the phenazine methosulfate+ascorbate system in the presence of Ca^{2+} led to cell shrinking, with the maximum effect being more than 20%. In contrast, under the same conditions, t-BHP caused cell swelling by up to 15%. Modification of the suspending medium (Ca^{2+} removing, clotrimazole addition, or enrichment with K^+) modulated the redistribution effects, suggesting that they were mediated to some extent by Gardos channel activation. These findings are important for understanding how oxidants modulate RBC cation channels.

1. Introduction

Circulating red blood cells (RBCs) are always subject to oxidative attacks. Their interaction with oxygen leads to hemoglobin autooxidation, resulting in the formation of superoxide radical (O_2^-) and other reactive oxygen species (ROS), mostly hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$) [1,2]. RBCs can also be attacked by exogenous ROS, originating from other blood cells (platelets, monocytes, neutrophils, and macrophages), as well as from vessel endothelium [2–4]. Imbalance between ROS production and antioxidant cell defence has been reported to result in oxidative stress (oxidation of protein thiol groups, depletion of nonenzymatic antioxidants, and lipid peroxidation of membrane phospholipids) [5].

ROS generation and oxidative stress in RBCs are augmented in various disease conditions, such as sepsis, shock, burns, ischemia–reperfusion, and certain enzymo- and hemoglobinopathies [6,7]. In some cases (sickle cell disease, thalassemia, and renal insufficiency), pathology-related redox alterations in RBCs go along with anemia, the appearance of an abnormally high-density RBC subset, and reduced intracellular K^+ [8–10]. In addition, loss of deformability and increased adhesiveness are observed, which lead to circulation disorders and may contribute to the development of ischemia and hypercoagulation [11].

Evidence exists suggesting that Ca^{2+}-activated K^+ channels (Gardos channels) are involved in the damaging RBC changes [12,13]. For example, the specific Gardos channel inhibitor clotrimazole is used in vivo to normalize the properties of RBCs in patients and various animal models [14]. On the other hand, in certain pathological situations, Gardos channel activation may be beneficial, preventing RBC lysis [15–17].

Oxidative stress contributes greatly to aging of normal RBCs in the circulation. With aging, intracellular ionic calcium progressively increases; cell volume and surface area become smaller (although the surface-to-volume ratio remains constant). It is not unlikely that cell aging also involves Gardos channel activation [18,19]. So, oxidative stress-induced changes in the properties of RBCs have not only clinical, pathophysiological relevance, but also physiological, regulatory relevance [20]. However, the physiological and pathophysiological functions of oxidative stress activation of Gardos channels remain obscure.

The response to oxidative stress at cellular and subcellular levels is studied using a variety of oxidative systems. For example, phenazine methosulfate (PMS) and t-butyl-hydroperoxide (t-BHP) are often employed. When applied to deoxygenated RBCs or RBCs treated with ascorbate at millimolar concentrations, PMS causes intracellular Ca^{2+} to rise and dramatically increases membrane permeability for K^+ by activating Gardos channels, which results in RBC dehydration [21,22]. The response to t-BHP is similar in

* *Abbreviations:* RBCs, red blood cells; ROS, reactive oxygen species; PMS, phenazine-methosulphate; t-BHP, tert-butyl-hydroperoxide; HEPES, 2-(*N*-2-hydroxyethylpiperazin-*N*'-yl)-ethanesulphonic acid); DMSO, dimethyl sulfoxide; EGTA, ethylene glycol-bis (h-aminoethyl ether)-*N,N,N V,N V*-tetraacetic acid; Hct, hematocrit.

Corresponding author. Tel.: +7 495 6123522; fax: +7 495 6128870.
E-mail address: irene_l@mail.ru (I.L. Lisovskaya).

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general; however, unlike PMS, t-BHP (1–3 mM) also considerably raises intracellular Na^+, which causes RBCs to swell and lose filterability [23,24]. On the other hand, Lang et al. reported that t-BHP induced RBC shrinkage [25].

This study compared PMS+ascorbate and t-BHP in terms of their effects on the volume-dependent parameters of normal human RBCs. Incubation with both oxidative systems resulted in the RBC density and osmotic resistance distribution shifts. Clotrimazole or Ca^{2+} added into the medium, as well as high extracellular K^+ levels, modulated the redistribution effects, suggesting that the effects observed are mediated to some extent by Gardos channel activation.

2. Materials and methods

2.1. Chemicals

All chemicals were of analytical grade. PMS, ascorbic acid, t-BHP, HEPES, glucose, DMSO, clotrimazole, and A 23187 were obtained from Sigma (St Louis, MO) or Sigma-Aldrich.

2.2. Preparation of RBCs

Freshly drawn blood (obtained from normal donors after informed consent) was anticoagulated with a citrate solution in a blood-to-citrate ratio of 9:1. Immediately thereafter, red cells were isolated and washed two times in 10 mM HEPES (pH 7.4) containing 5 mM KCl, 0.8 mM $MgSO_4$, 5 mM glucose and NaCl at a concentration required to achieve isotonic osmolality (U=300 mOsm/kg) (buffer1). At each washing step, the buffy coat was removed. Washed RBCs were re-suspended in the same buffer1 to a hematocrit (Hct) of 40%. The suspension obtained was stored at 4 °C for no longer than 3 h.

2.3. Experimental design

The initial 40% suspension of washed RBCs was diluted to Hct=5% with 10 mM HEPES (pH 7.4) containing 5 mM KCl, 0.8 mM $MgSO_4$, 1.5 mM $CaCl_2$, 5 mM glucose, and NaCl at a concentration required to achieve U=300 mOsm/kg (buffer2). In experiments meant to study the role of calcium ions, part of the samples were prepared using buffer1 supplemented with 1 mM EGTA (buffer3). In some experiments, a high-K^+ buffer was used. It contained 10 mM HEPES (pH 7.4), 100 mM KCl, 0.8 mM $MgSO_4$, 1.5 mM $CaCl_2$, 5 mM glucose, and NaCl at a concentration required to achieve U=300 mOsm/kg (buffer4). The stock solution of 100 mM ascorbic acid was prepared in isotonic NaCl and adjusted to pH 7.4 with NaOH. RBCs (5% suspension) were incubated with 10 mM ascorbic acid alone or in combination with 25–1500 μM PMS for 20 min at 37 °C with slow stirring. A 100 mM t-BHP stock solution was prepared in buffer1 from a 70% (wt/vol) commercial solution. RBCs were incubated with 1–3 mM t-BHP (final concentrations) for 30 min at 37 °C. Control cells were incubated with buffer2, buffer3 or buffer4.

2.4. Density distribution

The distribution of RBCs by their density was measured at room temperature using the phthalate method [26]. Mixtures of dimethylphthalate and dibutylphthalate were prepared to obtain a graded series of densities in the range from 1.066 to 1.144 g/ml. A drop of each mixture was taken into a microhematocrit capillary tube to a column height of 5–7 mm, and the capillary was then filled with the RBC suspension being studied (Hct, 40%), sealed, and centrifuged in a microhematocrit centrifuge at 12000 g for 6 min. The density distribution of an RBC sample was obtained by measuring the RBC column height above each phthalate mixture layer, relating it to the total column height (%), and plotting the result against the known phthalate mixture density.

2.5. Osmotic resistance distribution

The osmotic resistance of RBCs was determined using our modification of the profile migration method of Lew [22,27,28]. Briefly, light transmission was measured on a Thermomax microplate reader (Molecular Devices, Sunnyvale, United States) at room temperature. For these measurements, a graded series of buffered lysis media (25, 50, 75, 100, 125, 150, and 300 mOsm/kg) was prepared by mixing the isotonic buffer1 and the same buffer lacking NaCl (i.e., 25 mOsm/kg in osmolality) at appropriate ratios. The first horizontal row of flat-bottom wells contained distilled water (300 μl/well); the second and consecutive rows, lysis buffer (300 μl/well) in the order of increasing osmolality. A multipipette was used to distribute 6-μl aliquots of RBC samples (5% suspensions) so as to have each vertical row corresponding to one RBC sample. The final Hct in each well was 0.1%. The microplate was placed on an MS1 minishaker (IKA Werke, Staufen, Germany) for 30 min at room temperature. After incubation, 20% NaCl (20 μl/well) was added into the wells to bring their osmolality from the initial values of 0–150 mOsm/kg to 425–565 mOsm/kg, after which light transmission was immediately read at λ=650 nm. The intracellular hemoglobin concentration and, correspondingly, the RBC refraction index increase significantly on going into the hypertonic osmolality range but vary only slightly

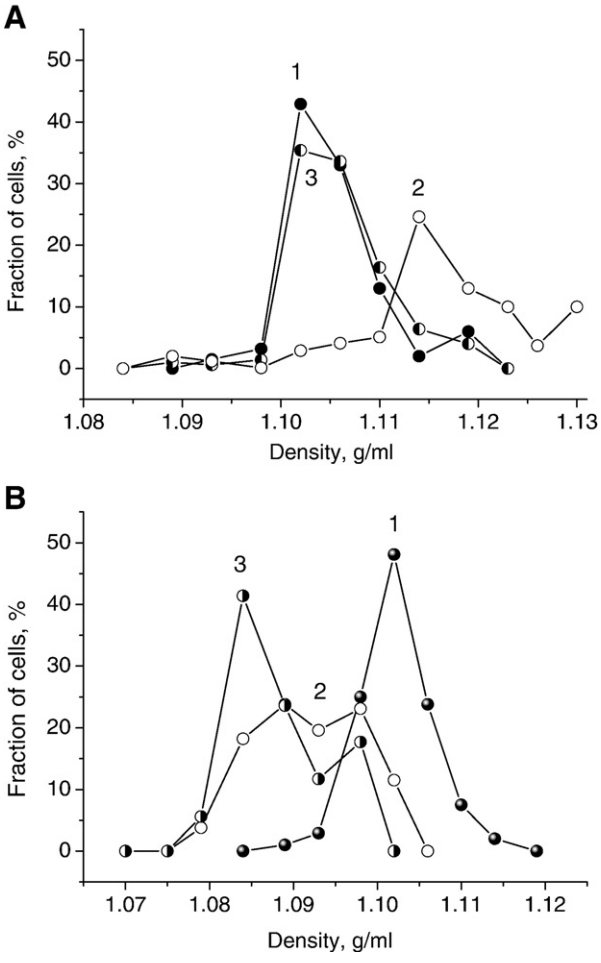

Fig. 1. Oxidative stress-induced shift in the RBC density distribution, as affected by the presence of Ca^{2+} in the medium: (A) 50 μM PMS+10 mM ascorbate and (B) 2 mM t-BHP. Cells resuspended in either (1, 2) in buffer2 (1.5 mM $CaCl_2$) or (3) buffer3 (1 mM EGTA) were incubated (1) without (control) or (2, 3) with the oxidative system for 20–30 min. Upon completion of the incubation, the cells were washed in isotonic buffer1, and the density distributions were determined with the phthalate method. The results shown are representative of three other independent experiments.

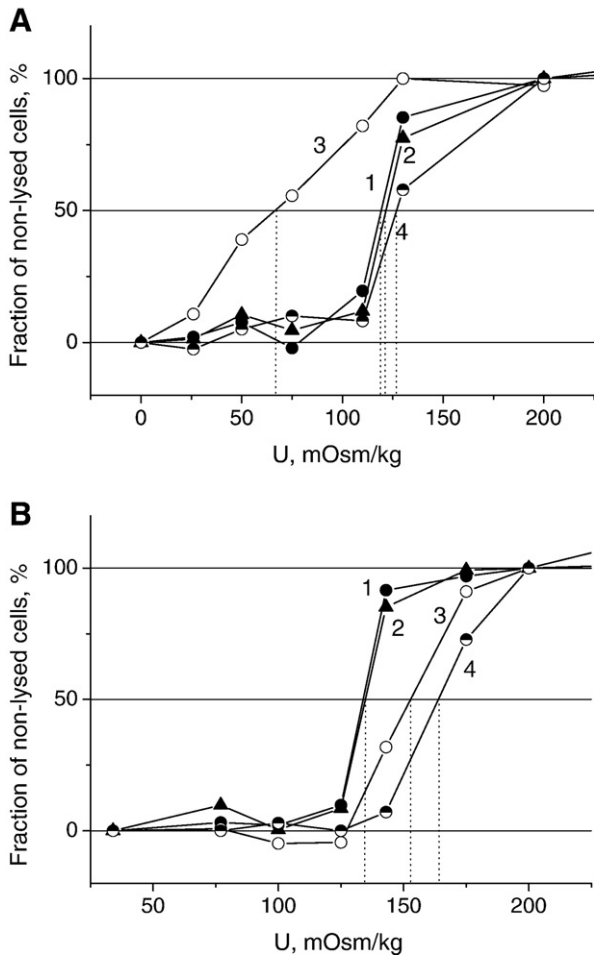

Fig. 2. Effect of Ca^{2+} on the magnitude of an oxidative stress-induced shift in the RBC osmotic resistance distribution: (A) 50 μM PMS+10 mM ascorbate and (B) 2 mM t-BHP. Cells were resuspended in (1, 3) buffer2 (1.5 mM $CaCl_2$) or (2, 4) buffer3 (1 mM EGTA) and incubated (1, 2) in the absence (control) or (3, 4) in the presence of (A) 50 μM PMS+ 10 mM ascorbate for 20 min or (B) 2 mM t-BHP for 20–30 min. Thereafter, aliquots for recording the osmotic resistance curves were taken from each sample. Shown are the results of typical experiments.

within the range. Therefore, the light transmission is determined largely by the concentration of cells that escaped lysis. This method makes it possible to obtain osmotic lysis curves simultaneously for 12 RBC samples. The osmolality value at which 50% cells undergo lysis (M_c, mOsm/kg; centre of the osmotic resistance distribution of RBCs) was chosen as a quantitative index of osmotic resistance of RBCs.

2.6. Statistics

Experimental results are presented as single observations representative of at least three others, or as means±S.E.M. of *n* parallel observations. Where appropriate, comparisons were made using Student's paired *t*-test.

3. Results

3.1. Calcium-dependent effects of oxidation on RBC density distributions

Fig. 1 shows the results of typical experiments in which the effects of two different oxidants on the RBC density distributions were studied in Ca^{2+}-free and Ca^{2+}-containing media. Fig. 1A compares the density distributions of intact RBCs (curve 1) and their counterparts treated with PMS+ascorbate oxidative system in HEPES buffer containing 1.5 mM Ca^{2+} (curve 2) or 1 mM EGTA (curve 3). One can see that, in the presence of Ca^{2+}, this treatment induced a shift to the right in the RBC density distribution, suggesting that the cells became dehydrated. No oxidation-induced shift in the RBC density distribution was observed in the medium without Ca^{2+}. Fig. 1B demonstrates that RBCs incubated with t-BHP for 30 min exhibited a leftward shift in their density distribution, as compared with the control cells, irrespective of the presence of calcium in the medium. Interestingly, the magnitude of the leftward shift (cell swelling) was much greater in the medium without Ca^{2+}.

3.2. Calcium-dependent effects of oxidation on osmotic lysis curves of normal human RBCs

Dehydrated RBCs are known to undergo lysis at lower values of osmolality than normal cells. In contrast, to lyse swollen RBCs, higher osmolality values are required [27,28]. Fig. 2A shows the results of a typical experiment, in which osmotic lysis curves were recorded for control RBCs and RBCs treated with PMS+ascorbate for 20 min in

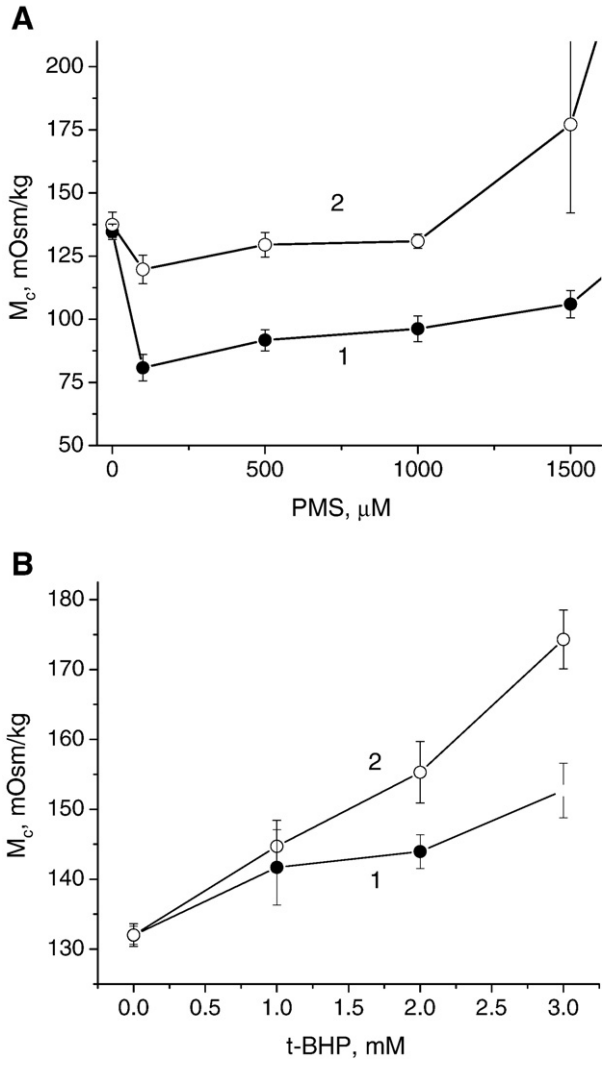

Fig. 3. Osmotic resistance index M_c versus oxidant concentration curves. Effect of clotrimazole. (A): Cells resuspended in buffer2 containing 10 mM ascorbate and varied concentrations of PMS were incubated for 20 min either (1) in the absence (control) or (2) in the presence of 10 μM clotrimazole. Thereafter, aliquots for recording the osmotic resistance curves were taken from each sample. (B): Cells resuspended in buffer2 with varied concentrations of t-BHP (1) in the absence or (2) in the presence of 10 μM clotrimazole for 30 min. Aliquots for recording the osmotic resistance curves were taken from each sample. Each data point (M_c, mOsm/kg) is the mean±S.E.M of 6–20 separate experiments.

HEPES buffer supplemented with either Ca^{2+} or EGTA. Fig. 2B is like 2A, except for the oxidant used (2 mM t-BHP). Obviously, oxidation treatment in a Ca^{2+}-containing medium changed the osmotic lysis curves. The osmolality values required for 50% lysis (M_c) of cells treated with PMS+ascorbate in the Ca^{2+}-containing medium were considerably lower than those determined for control cells, which is indicative of RBC dehydration (shrinking). In contrast, in the absence of Ca^{2+}, the osmotic lysis curves of treated RBCs did not differ from those of control RBCs (Fig. 2A). Fig. 2B demonstrates that, irrespective of the presence of calcium in the medium, the lysis curves of RBCs incubated with t-BHP are shifted into the hypertonic range relative to the control curves, suggesting cell swelling. However, the magnitude of the rightward shift was much smaller in Ca^{2+}-containing HEPES buffer.

3.3. Concentration-dependent effects of oxidants on the osmotic resistance distribution of RBCs

As shown earlier [22], shrinking of RBCs exposed to PMS+ascorbate in a Ca^{2+}-containing medium (Figs. 1A and 2A) results from oxidation-induced activation of their Gardos channels. Analysing Figs. 1B and 2B, one can see that the shifts in the density distribution and osmotic lysis curves of RBCs treated with t-BHP also depend on the presence of Ca^{2+}. RBCs swelled to a lesser extent if the medium contained Ca^{2+}. Given that t-BHP affects cell membrane permeability for cations and inhibits the RBC Ca^{2+}-pump, thereby raising intracellular Ca^{2+} concentration [29,30], it is reasonable to suggest that the t-BHP-induced Ca^{2+}-dependent RBC volume change observed in our experiments is related to Gardos channel activation. To test this suggestion, we compared how the osmotic resistance index M_c varied with oxidant concentration in a Ca^{2+}-containing medium in the presence and in the absence of the Gardos channel inhibitor clotrimazole. The results obtained are shown in Fig. 3.

As can be seen in Fig. 3A, PMS+ascorbate treatment caused a decrease in M_c (RBC shrinking). In a broad range of PMS concentrations, this effect was significantly smaller in the presence of clotrimazole. In contrast, t-BHP caused M_c to increase (RBC swelling) in a dose-dependent manner. Clotrimazole significantly potentiated the effect of t-BHP. Evidence suggesting the involvement of Gardos channels in oxidant-induced shifts in the volume-related parameters of RBCs was also obtained in experiments with RBCs suspended in a high-K^+ buffer. Gardos channel activation is not associated with cell swelling if transmembrane K^+ gradient is lacking [31].

As can be seen in Fig. 4, at high K^+ concentrations in the suspending medium, the effect of RBC shrinking in the presence of PMS+ascorbate is nearly abolished; incubation with t-BHP causes RBCs to swell to a much greater extent.

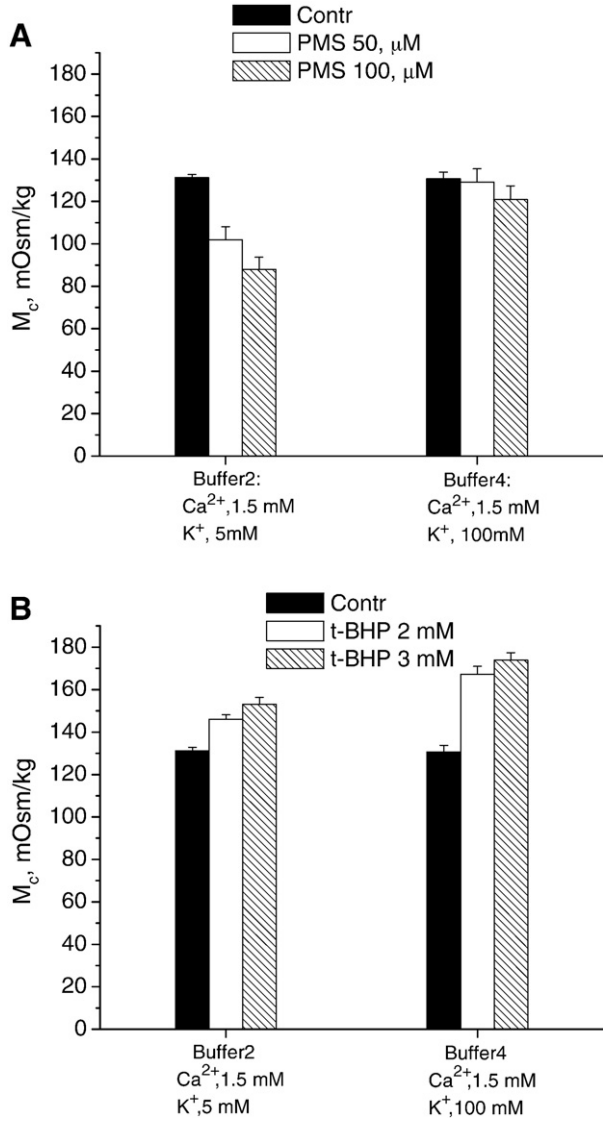

Fig. 4. Effect of K^+ at high concentrations on the magnitude of an oxidative stress-induced shift in the RBC osmotic resistance distribution. Cells were resuspended in buffer2 (5 mM K+) or buffer4 (100 mM K^+) and incubated with (A) 10 mM ascorbate+50 μM or 100 μM PMS or (B) 2 mM or 3 mM t-BHP. Thereafter, aliquots for recording the osmotic resistance curves were taken from each sample. Each data point (M_c, mOsm/kg) is the mean±S.E.M of 4–15 separate experiments.

4. Discussion

The mechanisms whereby PMS+ascorbate and t-BHP induce oxidative stress in RBCs are different. The primary response of RBCs to PMS+ascorbate is the generation of superoxide radicals [32,33], whereas the first to form during incubation of RBCs with t-BHP are alkoxyl and peroxyl radicals of hydroperoxides [34]. Both oxidative systems were shown to produce similar effects on the cells: reduced glutathione is rapidly depleted, hemoglobin undergoes oxidation, and passive K^+ permeability of cell membranes increases [22,24,34–37]. This study examined the effects of two oxidants – phenazine methosulfate (50–1500 μM)+10 mM ascorbate and t-BHP (1–3 mM) – on the volume-related characteristics of normal human red blood cells: their density (Fig. 1) and osmotic resistance (Figs. 2–4) distributions. A 20–30-min incubation at 37 °C with the oxidants studied produced opposite effects on the cell volume. If the suspending medium contained Ca^{2+}, incubation of RBSs with PMS+ascorbate led to a considerable increase in their density (shrinking), whereas a reduction in their density (swelling) was observed after incubation with t-BHP. If the suspending medium contained no Ca^{2+}, PMS+ascorbate failed to induce RBC shrinking (Figs. 1A and 2A), whereas the t-BHP-induced swelling was much more pronounced (Figs. 1B and 2B).

As shown in our earlier study, PMS+ascorbate causes RBCs to shrink in a Ca^{2+}-containing medium by activating the Gardos channel [22]. Suspecting that the reduction in the extent of t-BHP-induced swelling in a Ca^{2+}-containing medium is also due to the Gardos effect, we studied whether the PMS+ascorbate and t-BHP-induced osmotic resistance distribution shift is affected by (i) clotrimazole and (ii) high extracellular K^+. The data shown in Figs. 3 and 4 provide evidence suggesting the involvement of Gardos channel activation in the changes of the volume-related characteristics of RBCs induced by the oxidants used.

In our experiments with oxidation treatment, no vesiculation was observed (data not shown), suggesting that the cell surface area remained constant. Therefore, from the shift in M_c determined experimentally, it was possible to quantitatively estimate the change in the cell volume (see Appendix).

The maximum shrinking (minimum M_c of about 80 mOsm/kg) was observed at the PMS concentration of 100 μM and amounted to more than 20% of the volume of an intact RBC. With a further increase in the PMS concentration to 1.5 mM, the extent of shrinking decreased (Fig. 3A, curve 1) and partial hemolysis was observed. At a concentration of 10 μM, the specific inhibitor of Gardos channels clotrimazole largely abolished the shrinking effect PMS+ascorbate in a Ca^{2+}-containing medium. A statistically significant difference of M_c from its control value was obtained only for 100 μM PMS (Fig. 3A, curve 2). In the concentration range from 0 to 3 mM, incubation with t-BHP in a Ca^{2+}-containing medium for 30 min resulted in a dose-dependent RBC swelling. The greatest effect (volume increase of about 8%) was observed at a concentration of 3 mM. If the medium was either free of Ca^{2+}, contained clotrimazole, or contained K^+ at a high concentration, the greatest effect was as large as 14–15% (Figs. 3B and 4B).

In conclusion, in our experiments the two oxidants studied produced opposite effects on the RBC density and osmotic resistance distributions. Specifically, PMS+ascorbate caused RBC shrinking, whereas incubation with t-BHP resulted in RBC swelling. However, in both cases, there was activation of Ca^{2+}-dependent K^+ channels (Gardos-effect). It is Gardos channel activation that mediates RBC dehydration in response to PMS+ascorbate treatment [22] and reduces the extent of t-BHP-induced swelling. The differences observed are obviously due to different mechanisms of action of PMS+ascorbate and t-BHP on ion channels of the RBC membrane [2,34]. In Ca^{2+}-containing media, PMS+ascorbate raises intracellular Ca^{2+} concentration in RBCs (probably by inhibiting Ca^{2+}-ATPase [29]), modifies and activates Gardos channels, causing the cells to shrink [21,22,33]. At the same time, there is considerable evidence that incubation of RBCs with t-BHP leads to activation of nonselective cation channels [38,39], causing not only intracellular Ca^{2+}, but also Na^+ to rise. As a result, swelling is observed, which is partially offset by the Gardos effect. These findings are consistent with the suggestion that Gardos-channel activation is some general property of the cell response to oxidative treatment, which provides for RBC volume regulation in vivo [12,22,40].

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Appendix

The relationship between RBC volume and medium osmolality can be adequately described as $V/90 = A \cdot 300/U + B$, where V is cell volume (μm^3), U is medium osmolality (mOsm/kg), B is a parameter depending on the mean corpuscular hemoglobin content, and A is a parameter describing the ion homeostasis system in RBCs. For normal conditions, $A = 0.56$ and $B = 0.44$ [41,42]. The RBC membrane is relatively inelastic. Therefore, osmotic lysis of a cell with surface area S occurs when its volume reaches a critical value V_{cr} equal to the volume of a sphere having surface area S. Let S be 135 μm^2 [42], then $V_{cr} = 147.6$ μm^3. In our experiments with oxidation treatment of RBCs, no vesiculation was observed (data not shown). Hence, there were no surface area or hemoglobin loss, and the treated cells did not differ from control ones in the critical volume: $V_{cr}/90 = 1.64$. Knowing M_c, we can estimate the mean volume of treated RBCs in an isotonic medium: $V_{iso} = (M_c/250 + 0.44) \cdot 90$ μm^3. For $M_c = 80$ mOsm/kg (Figs. 3A and 4A), $V_{iso} = 68.4$ μm^3, which is 24% less than the normal value. This estimate agrees well with our earlier data showing that the density of RBCs treated with A23187 increased by an average of 30% [40]. For $M_c = 175$ mOsm/kg (Figs. 3B and 4B), $V_{iso} = 103$ μm^3, which is 14% greater than the normal value.

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