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Oxidation-induced calcium-dependent dehydration of normal human red blood cells

IRINA M. SHCHERBACHENKO, IRINA L. LISOVSKAYA, & VLADIMIR P. TIKHONOV

National Scientific Centre for Hematology, Novozykovsky pr. 4a, Moscow, Russia

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Abstract
Phenazine-methosulphate (PMS) is a strong oxidant that induces reactive oxygen species (ROS) formation in cells. Though it has been shown that PMS increases the red blood cell (RBC) membrane permeability to K^+, the hypotheses on the mechanism of PMS-induced effects are contradictory and there are no data on volume changes induced by this oxidant. Therefore, the influence of the PMS + ascorbate oxidative system on the volume of normal human RBCs was studied. In a Ca^{2+}-containing medium, PMS + ascorbate caused dehydration (shrinking) of RBCs judged by: (1) changes in the density and osmotic resistance distributions of RBCs, and (2) a decrease in their low-angle scattering assessed by FACS analysis. The dehydration resulted from activation of the Gardos channels, was PMS and ascorbate concentration-dependent, was associated with broadening of the density and osmotic resistance distributions of the RBCs, and decreased in the presence of the taxifolin and rutin antioxidants. These findings contribute to a better understanding of the physiology and pathology of oxidatively-modified RBCs and may be of practical significance in estimating the antioxidant activity of various substances.

Keywords: *RBCs, Phenazine-methosulphate, oxidation, Gardos channels*

Abbreviations: *RBC, red blood cell; PMS, phenazine-methosulphate; ROS, reactive oxygen species; FACS, fluorescence-activated cell sorting*

Introduction

Oxidation–reduction status is an important regulator of metabolic and physiological functions of the cell [1–3]. Constant interactions with oxygen cause red blood cell (RBC)-contained hemoglobin to undergo auto-oxidation, with the formation of superoxide radicals (O_2^-) and other reactive oxygen species (ROS), primarily hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^\cdot$) [4]. Other blood cells (platelets, monocytes, neutrophils and macrophages) and vessel wall endothelium serve as sources of exogenous ROS capable of attacking RBCs in the organism [5–7].

Under *in vivo* conditions, ROS formation and oxidative stress are increased in inflammation, shock, ischemia–reperfusion, and certain enzymo- and hemo-globinopathies; they are also stimulated by exposure to ionizing radiation [8–10]. Many pathological conditions are characterized by oxidative stress-provoked volume diminution and decreased deformability of RBCs, as well as shifts of their osmotic lysis curves to ranges of lower osmolality [11–13]. These changes are believed to arise from activation of Ca^{2+}-dependent K^+ channels (Gardos-effect), which results in K^+ efflux from RBCs, their dehydration, and volume decrease [14–16]. In particular, sickle cell disease is associated with episodic appearance in the blood of the patients of an RBC population with a high density and a low deformability [17]. Under normal conditions, oxidative stress induces RBC ageing, which is known to be accompanied by an increase in intracellular

Correspondence: I. M. Shcherbachenko, National Scientific Centre for Hematology, Novozykovsky pr. 4a, Moscow, Russia. E-mail: irascher@mail.ru

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ionized-calcium ($[Ca^{2+}]_i$) [18], a decrease in the cell volume and surface area [19,20], the disruption of the asymmetric distribution of phospholipids in the membrane layers [21], and other phenomena. Thus, changes in cation permeability of RBC membranes, frequently associated with oxidative stress, appear as a general phenomenon, which is observed under both pathological and normal conditions; apparently, it may have physiological (regulatory) implications, in addition to physio-pathological ones [22,23]. Oxidation-induced changes at the cellular and subcellular levels are the subject of increasing research interest, which stems, at least partially, from the search for antioxidants effective in protecting RBCs against oxidative stress [24–26]. Nevertheless, the mechanisms underlying oxidation-induced metabolic and functional changes in cells and their components remain poorly understood.

Experimental approaches to studying these changes have involved diverse oxidizing systems that induce ROS formation in cells and tissues, including phenazine-methosulphate (PMS). PMS is a strong oxidant that increases dramatically the permeability of RBC membranes to K^+; whether ascorbate is present [27–29] or absent [30–32]. Although the above effects are indicative of the ability of PMS to induce RBC dehydration, we are unaware of any reports in the literature on the directly measured volume changes induced by this oxidant in normal RBCs.

In this work, we studied the effects of the PMS—ascorbate oxidizing system on normal human RBCs. We demonstrate that incubation with the system results in a concentration-dependent hemoglobin oxidation and RBC dehydration. The dehydration was mediated by Gardos channel activation and associated with broadening of the density and osmotic resistance distributions of RBCs. Pre-incubation of RBCs with the well-known antioxidants taxifolin and rutin was shown to diminish these effects.

Methods

Chemicals

All chemicals were analytical grade. PMS, ascorbic acid, HEPES, glucose, DMSO, clotrimazole, and A 23187 were obtained from Sigma (St Louis, MO, USA)

Preparation of red blood cells

Freshly drawn blood (obtained from normal donors after informed consent) was citrated (the volumetric ratio of citrate solution to blood was equal to 1:9) or heparinized. Immediately thereafter, red cells were washed two times with HEPES buffer-1 (the buffy coat was removed) and resuspended in the same buffer to a hematocrit (Hct) of 40%. The suspension obtained was stored at 4°C for no longer than 3 h. HEPES buffer-1 contained 10 mM HEPES, 5 mM KCl, 0.8 mM $MgSO_4$ and an amount of NaCl required to get isotonic osmolality (*U*), pH 7.4.

Experimental design

The initial 40% suspension of washed-RBCs was diluted with HEPES buffer-2 to Hct = 5%. HEPES buffer-2 contained 10 mM HEPES, 5 mM KCl, 0.8 mM $MgSO_4$, 1.5 mM $CaCl_2$, 5 mM glucose, and an amount of NaCl required to satisfy the condition $U = 300$ mOsm/kg pH 7.4. In experiments where the role of calcium ions was studied, part of the samples was prepared by resuspending the RBCs in a calcium-free HEPES buffer-1 supplemented with 5 mM glucose and 1 mM EGTA. The stock solution of ascorbic acid (100 mM, pH 7.4) was prepared in HEPES buffer-1. RBC suspensions were incubated (37°C) under continuous slow stirring in the presence or in the absence of 10 mM ascorbic acid and 25–1500 μM PMS (final concentrations) for 20 min. Suspensions (5%) of intact RBCs, supplemented with the corresponding amounts of HEPES buffer-1 or -2 (with or without ascorbic acid) served as controls.

Density distribution

The distribution of RBCs by their density was measured using the phthalate method at room temperature [33]. Mixtures of dimethylphthalate and dibutylphthalate with densities in the range 1.066–1.144 g/ml were prepared. A drop of each mixture was taken into microhematocrit capillaries (corresponding to a column of 5–7 mm in each case), after which the capillaries were filled with RBC suspensions under study (Hct, 40%), sealed, and centrifuged in a microhematocrit centrifuge at 12,000*g* for 6 min. A density distribution of an RBC sample was obtained by measuring, in each capillary, the height of the upper portion of the column (above the phthalate mixture layer) and relating it to the total column height (%).

Osmotic resistance distribution and measurement of osmotic lysis

The osmotic resistance of RBCs was determined using our modification of the profile migration method of Lew [13,17,34]. Light transmission was measured on a Thermomax microplate reader (Molecular Devices, Sunnyvale, US) at room temperature. For these measurements, seven buffered solutions differing in osmolality were prepared (25, 50, 75, 100, 125, 150 and 300 mOsm/kg), by mixing at appropriate ratios the isotonic HEPES buffer-1 and a hypotonic NaCl-free HEPES buffer (osmolality, 25 mOsm/kg). The

first horizontal row of flat-bottom wells of the microplate was filled with distilled water (300 μl/well); each subsequent row contained one of the seven buffers (lysis media) in order of increasing osmolality (300 μl/well). Thereafter, a multichannel pipette was used to deliver 6-μl samples of RBC suspensions (Hct = 5%) into the wells in such a way that every vertical row corresponded to one RBC sample under study (12 samples/microplate).

The microplate was placed into an MS1 mini-shaker (IKA Werke, Staufen, Germany) and incubated (room temperature) under agitation for 30 min. When the incubation was complete, 20% NaCl was added into each well immediately prior to light transmission measurements (20 μl/well). As a result, the contents of the wells acquired osmolarities in the range 425–565 mOsm/kg (instead of the original 0–150 mOsm/kg, respectively). Light transmission was determined at $\lambda = 650$ nm. Because intracellular hemoglobin concentrations (and, correspondingly, the values of the refractive index of the RBCs) considerably increase with osmolality [35], and exhibit little differences within the new osmolality range, the light transmission value measured is actually determined by the concentration of the cells that resisted lysis.

This method makes it possible to obtain simultaneously osmotic lysis curves for 12 samples of RBC suspensions under study. The osmotic resistance of RBCs was assessed quantitatively by measuring the osmolality at which 50% cells undergo hemolysis (M_c, mOsm/kg; centre of osmotic resistance distribution of RBCs). In addition, the width of the distribution (W, mOsm/kg) was measured for each curve using the formula $W = (P_{90} - P_{10})$, where P_{90} and P_{10} are osmolality levels corresponding to a content of non-lysed cells, equal to 90 and 10%, respectively.

Table I shows the parameters of osmotic lysis curves of control and A23187-treated RBCs obtained using two methods. Method I (a modification of the profile migration method) is based on determining the percentage of cells that escaped lysis (by measuring light transmission of suspensions at $\lambda = 650$ nm). Method II is the standard technique used in osmotic resistance measurements, which is based on determining the concentration of free hemoglobin in suspensions. The data obtained indicate that the results obtained using these two methods match well.

Table I. Parameters of osmotic lysis curves of control and A23187-treated RBCs obtained using two methods.

	Sample	M_c (mOsm/kg)	W (mOsm/kg)
Method I	Control	133.5	35
	A23187	113.1	73.1
Method II	Control	134.2	28
	A23187	113.4	71

Washed RBCs from heparinized blood were resuspended in autologous plasma (Hct = 20%) and incubated with or without 10 μM ionophore A23187 at 37°C for 20 min. Thereafter, the cells were washed twice and resuspended in HEPES buffer-1. Control and ionophore-treated cells were introduced into eppendorf tubes containing lysis media of variable osmolality (900 μl/tube); the final Hct value equaled 0.1%. The tubes were incubated in a shaker (room temperature) for 30 min. (Method I). Each suspension was introduced into vertical rows 1 and 2 of the microplate (300 μl/well), prefilled with 20% NaCl (20 μl/well). The microplate was vigorously shaken, and the percentage of cells that escaped lysis was determined in each well by measuring the optical density at $\lambda = 650$ nm. (Method II). Eppendorf tubes with residual suspension were centrifuged for 5 min at 7000 rpm, and the supernatant was introduced into vertical rows 3 and 4 of the microplate (300 μl/well). The percentage of hemoglobin content in the supernatant was determined by measuring the optical density at $\lambda = 405$ nm. The results of a typical experiment that are representative of three further experiments are presented in the table.

Measurement of RBC volume distribution by flow cytometry

Fluorescence-activated cell sorting (FACS) analysis was performed on a flow cytometer (Becton–Dickinson, Immunofluorometry systems, Mountain View, CA, USA). Following incubation with PMS + ascorbate or A23187, the RBC suspension (Hct = 5%) was diluted 500-fold with HEPES buffer-1 HEPES buffer-2, to the concentration of 10^6 cells/ml. The cell size was assessed by the value of the small angle scattering (FSC-H, forward scatter height).

MetHb measurement

Methemoglobin (MetHb) and total hemoglobin (Hb_t) were measured in lysates of 5% RBC suspensions. The lysates were obtained by freezing (−80°C) and thawing the samples, followed by centrifugation to remove cellular debris. Thereafter, supernatant was diluted 60-fold by 15 mM phosphate buffer (pH 6.6) or the Drabkin solution, respectively, for measurement of MetHb (at $\lambda = 630$ nm; $\varepsilon = 4.4\ mM^{-1}\ cm^{-1}$) and total Hb ($Hb_t$) (at $\lambda = 540$ nm; $\varepsilon = 11\ mM^{-1}\ cm^{-1}$). The content of MetHb in the sample was determined as the ratio $[MetHb]/[Hb_t] \times 100(\%)$.

Statistics

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

Results

Dehydration of RBCs, caused by PMS + ascorbate: Osmotic resistance distribution

Osmotic lysis of dehydrated RBCs is known to occur at lower values of osmolality than those at which normal

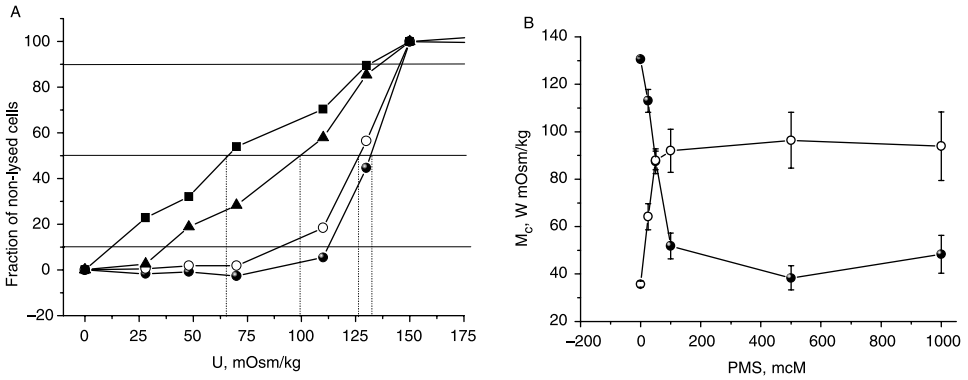

Figure 1. Dependence of osmotic lysis curves of normal human RBCs on PMS concentration. RBCs were incubated (37°C) with 10 mM ascorbate and variable concentrations of PMS (or without them, in control samples) for 20 min. Thereafter, aliquots for recording the osmotic resistance curves were taken from each sample. (A) oxidation-induced shift of the osmotic resistance curves (the graphs, corresponding to one series of measurements, are representative of eight further experiments): Control (●); PMS, 25 μM (○); PMS, 50 μM (▲); PMS, 100 μM (■). (B) effect of PMS concentration on the position of the center M_c (●) and the width W (○) of the osmotic resistance distribution. M_c and W (mOsm/kg) are means ± SEM, $n = 10–30$ different experiments.

cells are lysed [13,17,34]. Figure 1A shows the results of a typical experiment, in which osmotic lysis curves were recorded for RBCs incubated in HEPES buffer-2 in the presence of 10 mM ascorbate and variable concentrations of PMS. It is clear from the figure that the character of the curves changes as PMS concentration is increased: the osmolality values corresponding to hemolysis of 50% cells (M_c) decrease considerably, which is indicative of RBC dehydration (shrinking). As the curves migrate to the range of lower osmolality, they become more and more flat. The dependence of averaged parameters characterizing osmotic lysis curves on PMS concentration is shown in Figure 1B. It is clear M_c decreases significantly as the concentration of PMS increases from 0 to 100 μM, showing little changes at higher values. Conversely, the mean distribution width, W, significantly increases with RBC dehydration, changing in counterphase with M_c. Note that, at PMS concentrations in excess of 1500 μM, RBCs resuspended in isotonic medium undergo lysis (data not shown).

Involvement of ascorbate in RBC dehydration and hemoglobin oxidation

When RBCs were incubated with PMS (at concentrations up to 1000 μM), either without ascorbate or at concentrations not to exceed 5 mM, M_c was not changed, although hemoglobin oxidation did take place (Figure 2A, curves 1, 2 and 5). At 10 mM ascorbate, PMS-induced RBC shrinking, and the oxidation of hemoglobin was more intense (Figure 2A, curves 3 and 4). Figure 2B shows the dependence of the mean percentage of MetHb content in RBCs (incubated in the presence or in the absence of ascorbate) on PMS concentration. Clearly, the

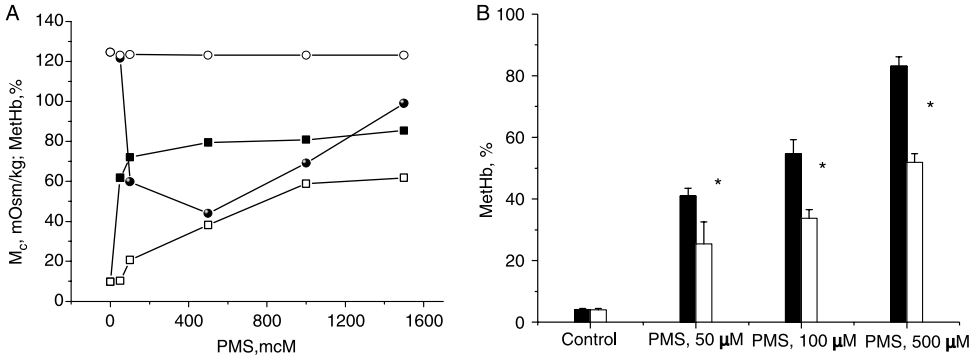

Figure 2. Effects of the concentration of PMS and the presence of ascorbate on the osmotic resistance distribution of RBCs and hemoglobin oxidation. Cells were incubated in HEPES buffer-2 (37°C) with 10 mM ascorbate and variable concentrations of PMS (or without them, in control samples) for 20 min. (A) dependences of the position of the centre of the osmotic resistance distribution of RBCs, M_c (○, ●) and MetHb accumulation (■, □) on PMS concentration in the absence (○, □) or in the presence of 10 mM ascorbate (●, ■). The graphs, corresponding to one series of measurements, are representative of four additional experiments. (B) dependence of MetHb accumulation in RBCs on PMS concentration in the absence (open bars, $n = 5$) or in the presence (black bars, $n = 12$) of 10 mM ascorbate. The content of MetHb is expressed as the percentage of the total content of hemoglobin; each value is mean ± SEM ⋆ Significant difference ($P < 0.05$) between the groups treated with PMS alone and PMS + ascorbate.

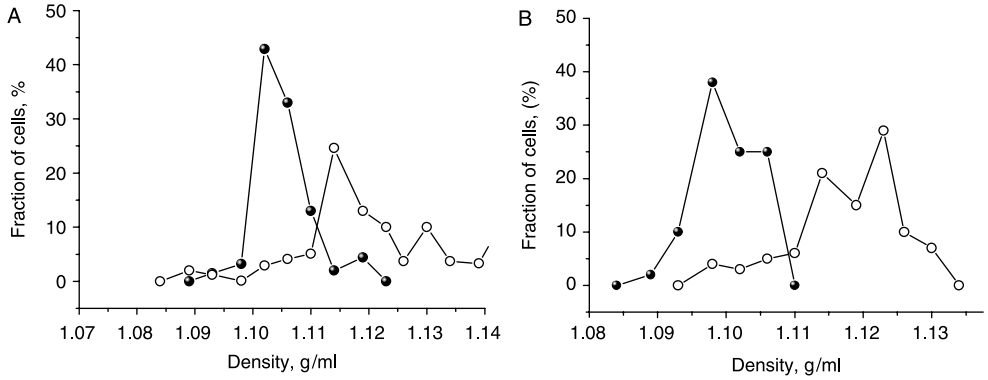

Figure 3. Effect of PMS + ascorbate or A23187 treatment on the density distribution of normal human RBCs. (A) cells were incubated in HEPES buffer-2 (37°C) in the absence (control, ●) or in the presence of 50 μM PMS + 10 mM ascorbate for 20 min (○). (B) identical to A, but the cells were incubated in the absence (control, ●) or in the presence of 4 μM ionophore A23187 (○).

presence of millimolar concentrations of ascorbate in the medium, prerequisite to RBC shrinking, increased significantly the content of MetHb in the cells. All subsequent experiments were carried out in the presence of 10 mM ascorbate.

Dehydration of RBCs, caused by PMS + ascorbate: Density distribution

The phenomenon of oxidation-induced dehydration of RBCs was further confirmed by the change in their density distributions. Figure 3A compares the density distributions of intact RBCs (curve 1) and their counterparts incubated in HEPES buffer-2 in the presence of the oxidative system PMS–ascorbate (curve 2). It is seen from the figure that the RBCs subjected to the oxidative treatment exhibit a shift in their distribution toward higher densities than those characteristic of the control. A density distribution of RBCs incubated in the same medium in the presence of 4 μM A23187 is shown in Figure 3B for comparison.

Flow-cytometric measurements of volume distribution of RBCs

Oxidation-induced changes in the volume distribution of RBCs were assessed by flow cytometry (FACS analysis). As evident from the forward scatter in FACS analysis histograms (Figure 4, 1–3), exposure to PMS + ascorbate led to RBC shrinking which increased with PMS concentration. A similar shrinking of RBCs is observed in incubation of the same suspension in the presence of 4 μM A23187 (Figure 4, histogram 4).

Dependence of RBC dehydration on the presence of Ca^{2+} in the medium

The obvious similarity of the effects of the oxidative system PMS–ascorbate to those induced by A23187 (Figures 3 and 4) suggests that oxidation-induced RBC dehydration is a consequence of the activation of Ca^{2+}-dependent K^+ channels (the Gardos channels) [14,34]. To validate this hypothesis, we explored the dependence of the effects observed on the presence in the medium of calcium ions and clotrimazole (a specific inhibitor of the Gardos channels). Figure 5 shows the dependence of the oxidation-induced RBC dehydration on the presence of Ca^{2+} in the medium. The shift in RBC density distribution, observed in the presence of Ca^{2+}, is not detected in a Ca^{2+}-free medium. The critical importance of extracellular Ca^{2+} for the oxidative dehydration of RBCs is also illustrated by Figure 5B.

The results of our flow-cytometric studies (Figure 6) provide yet another confirmation of the key role of calcium in oxidation-associated changes of the cellular volume.

Effects of clotrimazole

The effect of clotrimazole, a specific inhibitor of the Gardos channels, on oxidation-induced RBC dehydration is demonstrated in Figure 7. Clearly, the oxidation-induced dehydration was eliminated completely if RBCs were preincubated with 5–10 μM clotrimazole: both the density distribution curves (Figure 7A) and the parameters of osmotic lysis curves (Figure 7B) are the same as in the control.

Effects of antioxidants

Figure 8 demonstrates the effects of antioxidants on the osmotic resistance of RBCs treated with PMS + ascorbate. Cells were preincubated with 200 μM taxifolin or rutin (which is in the effective concentration range reported for flavonoids in RBCs earlier [36,37]) for 20 min at ambient temperature before oxidative treatment. It can be seen that either antioxidant reduces significantly the effects of

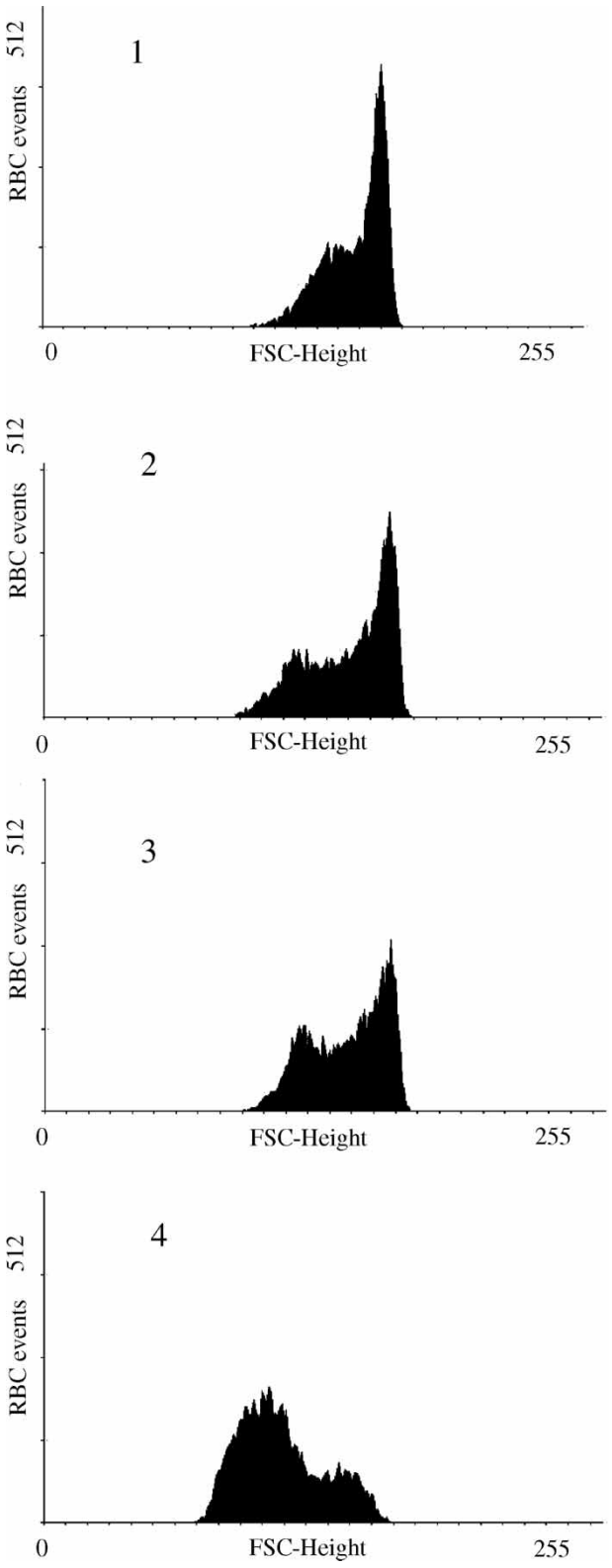

Figure 4. Effect of PMS + ascorbate or A23187 treatment on the volume distribution of normal human RBCs. The cells were incubated in HEPES buffer-2 (37°C) in the absence (1) or in the presence of 50 μM PMS + 10 mM ascorbate (2), 100 μM PMS + 10 mM ascorbate (3) or 4 μM ionophore A23187 for 20 min (4). The forward-scatter histograms are from a representative FACS analysis experiment. Geometric means of small-angle scattering of the cell samples: $Gm_1 = 148.2$; $Gm_2 = 140.2$; $Gm_3 = 138.2$ and $Gm_4 = 108.6$.

PMS + ascorbate on RBC shrinkage and suspension heterogeneity (width *W* of the osmotic resistance distribution).

Discussion

Oxidation-induced nonspecific increase in membrane permeability to monovalent cations are characteristic of many cell types [38]. Oxidative damage of RBC membranes frequently produces an increase in the cell volume, a decrease in osmotic resistance, and hemolysis [39,40]. However, some *in vivo* situations accompanied by oxidative stress, including sickle cell disease and RBC ageing, present with an opposite picture: the cell volume decreases, likely due to the Gardos channel activation [18,34].

PMS is known to be capable of *in vitro* interactions with hydrogen donors, such as NADH, with the formation of superoxide anions (O_2^-) and other ROS [27,30]. It was shown that RBC incubation in the presence of the ascorbate + PMS system increased the transmembrane K^+ fluxes [28,29]. Subsequent studies by Gibson et al. [31,32] demonstrated that the same changes in K^+ permeability of membranes were observed after incubation of deoxygenated-RBCs with PMS in the absence of ascorbate. The authors related the observed effects to the activation of two transport systems of RBCs by PMS, namely, the KCl-co-transporter and Ca^{2+}-activated K^+-channels (Gardos channels). In their experiments, Gibson et al. measured the cell volume and found no change in this parameter in RBCs subjected to oxidative stress. Although the measurement conditions were not fully specified, it is reasonable to suggest that they took measurements in a medium high in K^+, because their resuspending solution contained 80 mM K^+[32].

Three distinct methods were used in this work to demonstrate that treatment with PMS of normal human RBCs suspended in calcium-containing media in the presence of 10 mM ascorbate causes their dehydration (shrinking), which depends on PMS concentration. RBCs shrinking was frequently noted at PMS concentrations as low as 25 μM, attaining maximum at about 500 μM. It can be shown that maximum shrinking is comparable to the effect observed as a result of RBCs incubation with the ionophore A23187 [34,41].

The lack of any effect in calcium-free media (Figures 5 and 6) and clotrimazole inhibition (Figure 7) indicate that oxidation-induced RBC dehydration results from $[Ca^{2+}]_i$ elevation and the Gardos channel activation [34]. In a prior work, we demonstrated that factors increasing intracellular Ca^{2+} (RBC incubation with the ionophore A23187 in heparinized plasma or with the calcium pump inhibitor vanadate in a Ca^{2+}-containing medium, as well as ATP depletion) increase considerably the width of density and filterability distributions

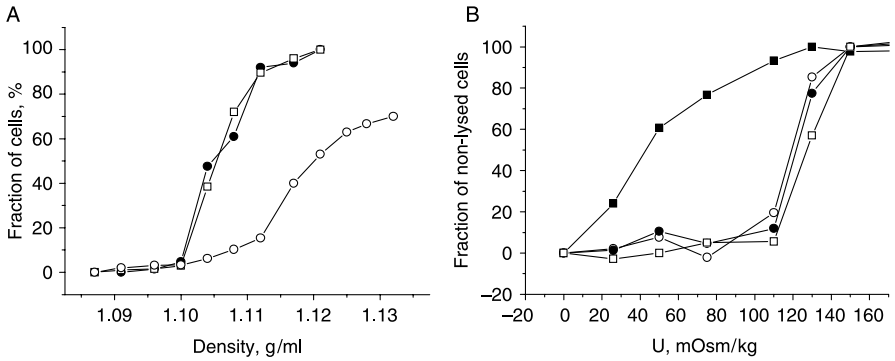

Figure 5. Dependence of oxidation-induced dehydration of normal human RBCs on the presence of Ca^{2+} ions. (A) cells were washed and resuspended in HEPES buffer-1 (□) or HEPES buffer-2 (● and ○), followed by incubation in the absence (control, ●) or in the presence of 50 μM PMS + 10 mM ascorbate for 20 min (□ and ○). Upon completion of the incubation, the cells were washed in isotonic HEPES buffer-1, and the density distribution was determined by the phthalate method. (B) cells were incubated in HEPES buffer-1 (●, □) or HEPES buffer-2 (○, ■), in the absence (●, ○) or in the presence (□, ■) of 100 μM PMS + 10 mM ascorbate, after which aliquots for determining the osmotic resistance distribution were taken.

of the cells [42]. In this work, we demonstrate that incubation with the oxidative system PMS + ascorbate also increases the heterogeneity of the population of normal human RBCs.

In this report, we examined the effect of two flavonoids, taxifolin and rutin, on RBC dehydration. Flavonoids are known to be antioxidants that scavenge free radicals, protect against lipid peroxidation and chelate metal ions. Therefore, flavonoids may have great relevance for prevention and therapy of diseases in which oxidants or free radicals are implicated [43,44]. The fact that preincubation of RBCs with

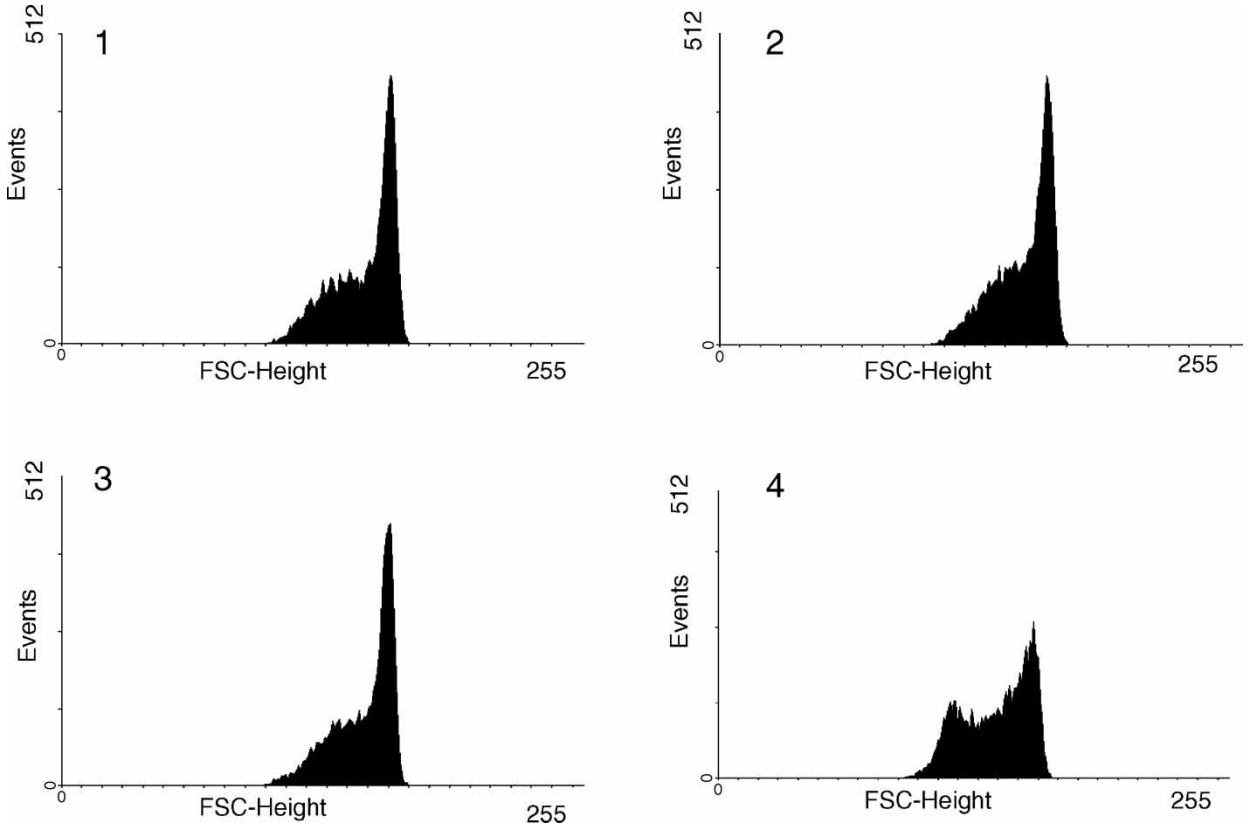

Figure 6. Effects of calcium on PMS + ascorbate induced volume distribution of normal human RBCs. Cells were resuspended in the Ca^{2+}-free HEPES buffer-1 (1, 3) or HEPES buffer-2, containing 1,5 mM Ca^{2+} (2, 4), followed by incubation in the absence (1, 2) or in the presence (3, 4) of 100 μM PMS + 10 mM ascorbate for 20 min. The forward-scatter histograms are from a representative FACS analysis experiment. Geometric means of small-angle scattering of the cell samples: $Gm_1 = 148.1$; $Gm_2 = 148.9$; $Gm_3 = 148.0$ and $Gm_4 = 139.05$.

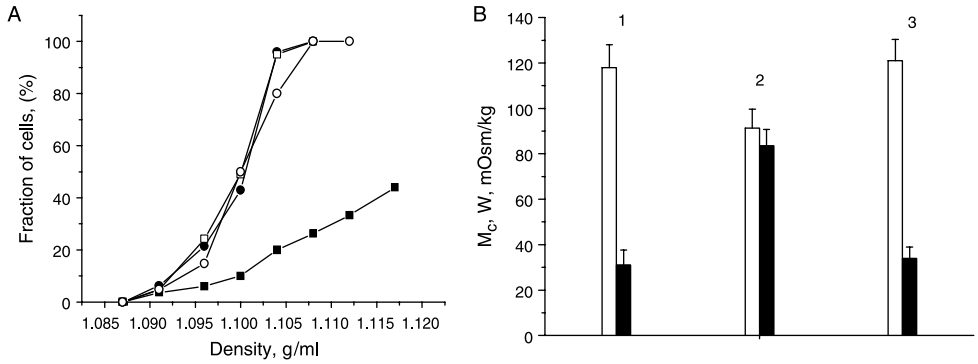

Figure 7. Effects of clotrimazole, an inhibitor of the Gardos channels on the oxidation-induced dehydration of normal human RBCs. (A) cells were first incubated (37°C) in the absence (●, ■) or in the presence of 10 μM clotrimazole (□, ○) for 15 min, and then, in the absence (●, □) or in the presence of 50 μM PMS + 10 mM ascorbate (○, ■), for additional 20 min. Upon completion of the incubation, the RBCs were washed in the isotonic HEPES buffer-1, and the density distribution was determined by the phthalate method. (B) cells were first incubated (37°C) in the absence (1, 2) or in the presence (3) of 10 μM clotrimazole for 15 min, and then, in the absence (1) or in the presence (2, 3) of 25 μM PMS + 10 mM ascorbate, for additional 20 min. Thereafter, aliquots were taken for measurements of osmotic resistance. The values of osmolality, corresponding to the position of the centre (M_c) and the width (W) of the distribution, were expressed as means ± SEM, $n = 3$ different experiments.

flavonoids reduces their dehydration induced by PMS + ascorbate (Figure 8) can be useful in assessing the potency of antioxidants.

Thus, (1) the extracellular Ca^{2+} dependence, (2) the amenability to inhibition by clotrimazole, and (3) the broadening of density and osmotic resistance distributions are indicative of the key role of the Gardos channel activation in the phenomenon of RBC shrinking, induced by the PMS + ascorbate oxidative system.

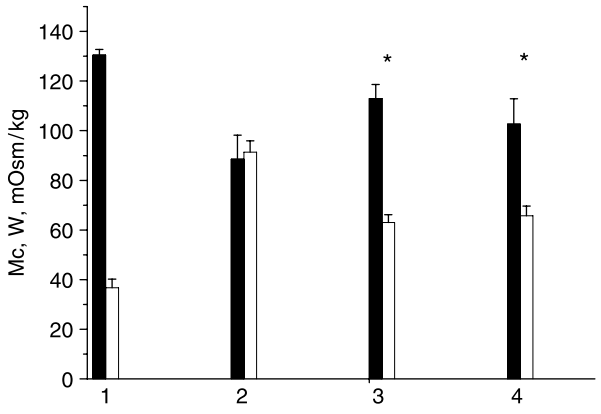

Figure 8. Effects of antioxidants taxifolin and rutin on the osmotic resistance distribution of RBCs treated with PMS + ascorbate. RBCs were first incubated at 22°C in the absence (1, 2) or in the presence of 200 μM taxifolin (3) or 200 μM rutin (4) for 20 min, and then, in the absence (1) or in the presence of 50 μM PMS + 10 mM ascorbate (2–4) at 37°C, for additional 20 min. Thereafter, aliquots were taken for measurements of osmotic resistance. The values of osmolality, corresponding to the position of the centre (M_c, black bars) and the width (W, open bars) of the distribution, were expressed as means ± SEM, $n = 6$ different experiments. * Significant difference ($P < 0.05$) between the groups treated with PMS alone and PMS + antioxidant.

Why does RBC incubation in the presence of the system PMS + ascorbate induce such a powerful activation of the Gardos channels, comparable in extent to the effects of the ionophore A23187?

In vitro experiments demonstrated that $[Ca^{2+}]_i$ elevation observed in the course of oxidative treatment of RBCs may be the consequence of inhibition of plasma membrane-associated Ca^{2+}-dependent ATPases (calcium pumps) [44,45]. In addition, it was shown that the sensitivity of the Gardos channels to Ca^{2+} strongly depends on the redox state of the plasma membrane [31,46]. It is conceivable that the effect is mediated by calpromotin (peroxyredoxin II), an oxidation-sensitive cytoplasmic protein capable of activating the Gardos channels on binding to the cytosolic surface of the plasma membrane [47,48]. This protein is known as a major factor of the endogenous antioxidant defense system, which protects RBCs from free-radical damage [49]. Yet another possible cause may lie in that the ascorbate–PMS system additionally activates the Gardos channels in a specific manner.

In our work, we studied the dehydration of oxygenated (air-exposed) RBCs by PMS in the presence of high ascorbate concentrations. We never observed PMS-induced RBC dehydration at ascorbate concentrations in the range from 0 to 5 mM. What is the role of ascorbate in the effects observed? It is well known that, in spite of its strong antioxidant properties, ascorbate may provoke oxidation [50]. Under our experimental conditions, ascorbate might have potentiated the oxidizing effect of PMS; this assumption is corroborated by an increase in the MetHb formation (Figure 2).

Unlike the use of ionophore A23187, the calcium pump inhibitor vanadate [51] or ATP depletion [43], oxidative treatment of RBCs may hold physiological

implications. There are some agents capable of interacting with RBCs *in vivo*, which activate the Gardos channels. They include PgE_2 [52] and cytokines [53], which act via specific receptors. The Gardos effect is the only mechanism known to date, whereby the volume of mature normal RBCs is decreased within minutes. It may protect RBCs from hemolysis, induced by complement activation [54] and attenuate cell damage in spherocytosis [23]. There is evidence to believe that the regulation of the volume of circulating RBCs is related to the Gardos effect [41].

In conclusion, this study is the first to directly address the dehydration of normal RBCs induced by PMS + ascorbate. Incubation of oxygenated-RBCs in a Ca^{2+}-containing medium in the presence of PMS and millimolar ascorbate induces both oxidation of hemoglobin and dehydration of the cells; the effects were PMS and ascorbate concentration-dependent. The dehydration, caused by activation of the Gardos channels was observed along with a broadening of the density and osmotic resistance distributions of RBCs. These effects were smaller in the presence of the antioxidants tested (rutin and taxifolin). The data obtained may contribute to a better understanding of the response of RBCs to oxidation under normal and pathological conditions and may be of practical significance in assessing the potency of various antioxidants.

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