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Sodium selenite as modulator of red cell shape

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Abstract

Addition of sodium selenite to human red cells, under ATP deplete conditions, induces a rapid oxidation of both glutathione and protein sulphydryl groups. Selenite also inhibits the discocyte-echinocyte shape transformation and stops the process before completion. Parallel to the effect on shape, selenite reduces the dephosphorylation of phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 4-monophosphate. Therefore our results support a shape change mechanism based on the metabolism of phosphoinositides and compatible with the bilayer-couple hypothesis.

Key words: Erythrocyte; Shape change; Membrane; Phosphoinositide metabolism; Phosphatidylinositol 4,5-bisphosphate; Phosphatidylinositol 4-monophosphate; (Human)

1. Introduction

Although the architecture of the red cell and its membrane-associated cytoskeletal network is known in detail [1–3] the factors that determine and control the characteristic biconcave shape are poorly understood. It appears that an intact membrane-associated skeleton is vital for normal shape, as several hemolytic disorders characterized by loss of biconcave shape, have been linked to defects in the membrane skeleton [4,5]. In some hemolytic anemias, such as hereditary spherocytosis, the cells not only become spherical but are also extremely fragile, implying that the membrane skeletons also furnish stability and elasticity to the cell [4]. Also the link between the skeleton and the membrane seems to be important for normal shape as scission of this linking protein prevents any shape changes and reduces deformability of red cell membranes [6].

Energy is also required for normal shape as the discoid shape is lost in favour of a spiculated and eventually a spherical form when the cell consumes its ATP [7]. This shape change is reversible up to the point preceding membrane loss and the discoid shape can be recovered if the cell is allowed to resynthesize ATP [8,9]. Under several different conditions of metabolic depletion it has been shown that the shape transformation parallels the dephosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP_2), phosphatidylinositol 4-monophosphate (PIP) and phosphatidic acid (PA), and not that of membrane skeletal proteins [10,11]. Since these phospholipids mainly are localized to the inner leaflet of the membrane [12], dephosphorylation of the phosphoinositides would reduce the area of that leaflet. Therefore shape changes due to metabolic depletion lends support to the bilayer-couple hypothesis that explains the cell shape as a function of the relative areas of the inner and outer leaflets of the membrane [13,14].

Recently it was shown that sodium selenite has a stabilizing effect on the red cell and it was inferred that this was due to altered interactions between the membrane lipids and the membrane skeleton as well as changes in skeletal protein conformation [15]. We have extended their study and investigated the effect of selenite on the metabolism of the phosphoinositides. The results show that sodium selenite not only stabilizes the cell, as found previously, but also is a potent echinocytic agent and that this effect can be linked to the turnover of phosphoinositides. In addition, selenite induces a rapid depletion of reduced glutathione (GSH) which leads to cross-linking of membrane skeletal proteins.

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Abbreviations: PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP, phosphatidylinositol 4-monophosphate; PA, phosphatidic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid.

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2. Materials and methods

Fresh human blood was kindly provided by the blood bank at the University Hospital, Umeå. Bovine serum albumin (essentially fatty acid free) and glutaraldehyde were from Sigma. Penicillin G-streptomycin mixture was from Gibco and ATP monitoring kit from LKB. Carrier-free $H_3^{32}PO_4$ from Amersham, Silica gel 60 thin-layer chromatography plates (20 cm × 20 cm) from Merck.

The red cells were washed five times with ice-cold buffer H (130 mM NaCl, 3.7 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, 25 mM Hepes (pH 7.5)) and twice with ice-cold complete buffer H (buffer H containing 100 units ml^{-1} penicillin G, 100 units ml^{-1} streptomycin and 1 mg ml^{-1} bovine serum albumin). Plasma and buffy coat were removed by aspiration after each centrifugation.

Red cells (10% hematocrit) were labelled with ^{32}P (0.74 MBq ml^{-1}) by incubation for 23–30 h at 37°C in complete buffer H supplemented with 10 mM glucose and 1 mM adenosine. After incubation the cells were washed four times with buffer H and twice with complete buffer H.

Metabolic depletion was initiated by resuspending ^{32}P-labelled cells to 10% hematocrit in complete buffer H, containing the indicated concentration of Na_2SeO_3. In some experiments, GSH was depleted by incubation in medium containing 1-chloro-2,4-dinitrobenzene (CDNB) [16].

Morphology. Erythrocytes were fixed by mixing 30 μl of the cell suspension with 0.2 ml 1% glutaraldehyde in 0.1 M potassium phosphate buffer (pH 7.5) for 60 min at room temperature. The extent of crenation was determined by phase contrast microscopy, counting at least 400 cells of each sample and classifying the cells in five classes of crenation [17].

ATP. ATP was determined by the luceferin-luciferase assay [18] as described previously [11].

GSH. For determination of GSH, 0.5 ml cell suspension was lysed in 1 ml cold water and 1 ml of this mixture was used immediately [19].

Hemoglobin. The concentration of haemoglobin in lysates was determined from measurements of absorbance at 539 nm using a molar absorbtivity of $13.4 \cdot 10^3$ $M^{-1} cm^{-1}$ per haem [20].

Phospholipids. Lipids were extracted from frozen samples of cell suspension using acidic methanol/chloroform and separated by thin-layer chromatography as described before [11]. Radiolabelled phospholipids were detected by autoradiography and quantified by liquid scintillation counting of scraped spots. Lipid phosphorus was determined spectroscopically [21].

Polyacrylamide gel electrophoresis in sodium dodecyl sulphate (SDS-PAGE). The isolated membrane fraction was analyzed on 7% slab gels according to Laemmli [22]. 2-Mercaptoethanol was omitted from the sample buffer to obtain non-reducing conditions. Occasionally bands from non-reducing gels were cut out, incubated in the presence of 2-mercaptoethanol and rerun on 7% SDS gels.

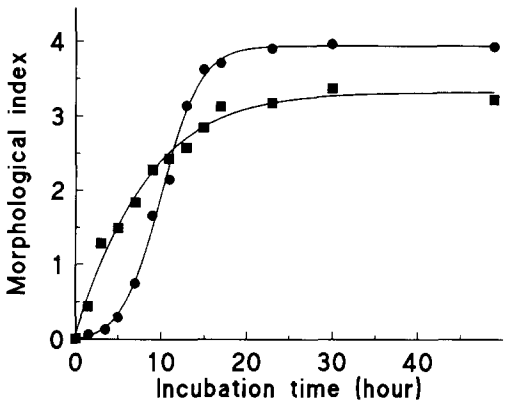

Fig. 1. Metabolic depletion of human red cells. Washed red cells were suspended at 10% hematocrit in complete buffer H and incubated at 37°C in the absence (●) and presence of 50 μM sodium selenite (■). At the indicated times, samples were withdrawn and the morphological index was determined as described in the text.

3. Results

When red cells were incubated under ATP-depleting conditions (i.e., in the absence of nutrients) at 37°C, the normal biconcave shape was gradually lost and the cells became increasingly echinocytic. Addition of sodium selenite to the incubation medium had two striking effects; the initial rate of the shape change (during the early phase) was much quicker and the shape change process levelled of at a lower final morphological index, as shown in Fig. 1.

Previously, we and others [10,11] have suggested that phosphoinositide metabolism is an obligatory step in metabolically induced shape changes. Since selenite both increased (during the initial phase) and decreased (during the late phase) the rate of echinocytosis, selenite should according to the hypothesis also influence the turnover of the phosphoinositides. Thus, the shape change behaviour suggested that selenite initially should increase the dephosphorylation of PIP_2, PIP and PA, whereas at later stages the metabolism of these phospholipids should be inhibited, resulting in a less extensive dephosphorylation compared to control cells. To investigate this, red cells were labelled by preincubation for about 24 h with nutrients and [^{32}P]orthophosphate, a treatment that incorporates the label into PIP_2, PIP and PA as well as several proteins [10,11,23,24]. These ^{32}P-labelled cells were then metabolically depleted in the absence and presence of selenite, while shape and turn-over of the label were monitored.

Although labelled cells went through similar shape changes and reached the same final morphological index as unlabelled cells, the overall process was faster (cf., Fig. 1 and Fig. 2). Morphological indexes of 1 and 2 were reached after ~4 and ~6.5 h depletion, respectively, compared to ~7.5 and ~10 h required for unlabelled cells. Since we have observed this behaviour several times before it seemed likely that the labelling process (i.e., 24 h incubation) somehow sensitized the cells to depletion. Again selenite reduced the final morphological to ~3 but the effect on the initial rate of shape change was not as significant as in the case of unlabelled cells (Fig. 2).

In accordance with earlier observations [11,23] only PIP_2, PIP and PA incorporated the ^{32}P-label among the phospholipids. The specific activity of the total phospholipid fraction should therefore be directly proportional to the amount of the phosphoinositides. The specific activities of the lipid fraction of selenite-treated cells were higher than that of untreated cells, indicating that selenite reduced the rate of dephosphorylation of the phosphoinositides. Since the intracellular level of ATP was unaffected it seemed quite unlikely that selenite would have increased the incorporation of label into the phosphoinositides.

In control cells, the levels of PIP and, in particular, PIP_2 decreased whereas the level of PA was more or less unaffected by ATP depletion (Fig. 3), in accordance with previous measurements [10,11]. Addition of selenite to the incubation medium had two major effects on the phosphoinositide turn-over. Firstly, the rate of dephosphorylation of PIP_2 decreased considerably; after 25 h depletion, ~26% of the initial PIP_2 remained in cells incubated with 50 μM selenite compared to ~10% in untreated cells. Secondly, the level of PIP increased to about 170–180% of the initial level

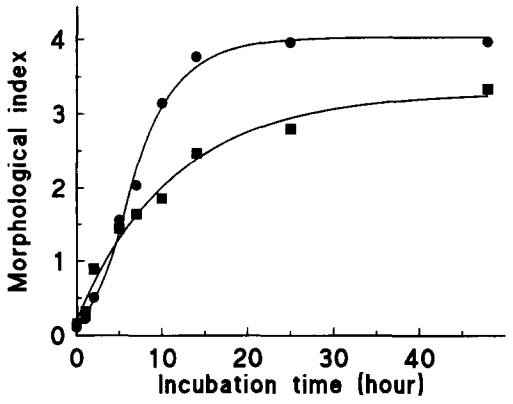

Fig. 2. Metabolically induced shape changes of ^{32}P-labelled human red cells. Washed red cells were equilibrated with [^{32}P]orthophosphate before initiating metabolic depletion in the absence (●) and presence of 50 μM sodium selenite (■). At the indicated times, samples were withdrawn and the morphological index was determined as described in the text.

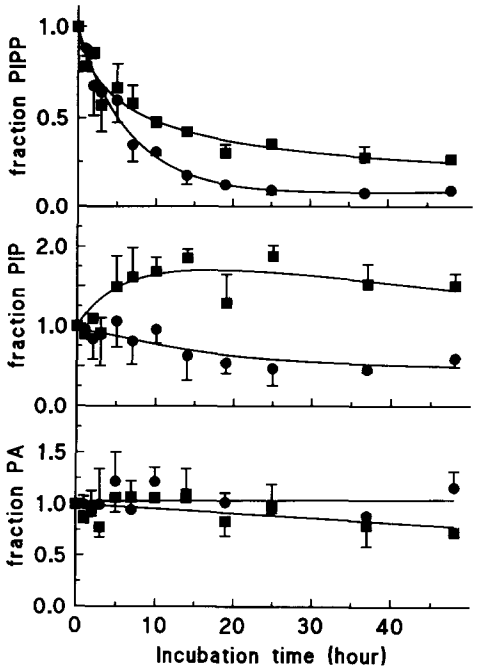

Fig. 3. Metabolism of the phosphoinositides during metabolic depletion. Red cells were labelled with ^{32}P, washed free of phosphate and suspended in complete buffer H. Samples were withdrawn and analyzed as described under Materials and methods, and the fractions of PIP_2, PIP and PA in the absence (●) and presence of 50 μM sodium selenite (■) were determined. (The data depicted in Fig. 2 was also obtained from this experiment).

in selenite-treated cells, in striking contrast to the control cells in which the level of PIP gradually decreased upon depletion. The level of PA appeared not to be influenced to any larger extent by selenite. Lower concentrations of selenite affected the turn-over of the phosphoinositides in a similar way but to a lesser extent. At 10 μM selenite, the level of PIP_2 had dropped to ~15% after 25 h depletion whereas the level of PIP appeared to be unaffected. Again PA did not change significantly during the incubation.

In contrast to the intracellular level of ATP, GSH was profoundly affected by selenite as can be seen in Fig. 4; at 10 μM selenite the level was halved in less than 1 h and at 50 μM all GSH was consumed more or less immediately. Control experiments showed that selenite oxidized GSH also in a cell free system, as been noted before [25]. When selenite was added to a GSH solution (i.e., the remaining supernatant of lyzed cells) before precipitation of protein sulphydryls, the GSH level was decreased in a manner dependent on the concentration of selenite.

However, when added to the same GSH solution after precipitation and neutralization, selenite did not, oddly enough, cause any oxidation of GSH.

In another experiment cells were incubated in the presence of glucose and thus allowed to resynthesize any consumed ATP or NADPH. In this case selenite

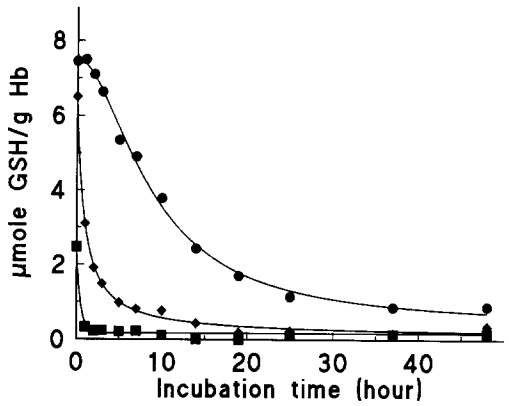

Fig. 4. Metabolism of glutathione during metabolic depletion. Washed red cells were suspended at 10% hematocrit in complete buffer H and incubated at 37°C in the absence (●) and presence of 10 µM (◆) and 50 µM (■) sodium selenite. At the indicated times, samples were withdrawn and the concentration of glutathione was determined.

reduced the intracellular level of GSH immediately after addition but upon incubation the normal level of GSH was restored. Assuming that selenite reacts similarly under ATP replete conditions, this result indicated that any oxidized glutathione can be reduced again and that selenite did not interfere with this reduction nor with the generation of the NADPH (mainly by the pentose phosphate pathway) needed to reduce the oxidized glutathione.

In addition to the effect on phosphoinositide and GSH metabolism, incubation with selenite under ATP depleting condition led to a striking colour change of the red cell suspension, implying that depletion of GSH induced formation of methemoglobin. However, after 10 h incubation 50 µM selenite had only induced formation of 5% methemoglobin. Removal of GSH by CDNB, which is known to result in rapid oxidation of hemoglobin to methemoglobin [16] produced 40–50% methemoglobin after 10 h depletion.

Similar to several studies of GSH depleted red cells [26,27], analysis of the membrane fraction by SDS-PAGE under non-reducing conditions showed an increased formation of high molecular weight complexes as well as an increased amount of globin in samples obtained from selenite-treated cells. Reanalysis, under reducing conditions (i.e., in the presence of 2-mercaptoethanol) indicated that the high molecular weight complexes contained mainly spectrin. The increased globin-content in selenite-treated cells implied that depletion of GSH may not only oxidize hemoglobin to methemoglobin but also to hemichromes that attach (as Heinz bodies) to the red cell membrane [28,29]. It is also possible that globin may attach covalently to spectrin and thus to the membrane fraction, as has been suggested upon peroxidation [30].

4. Discussion

Selenium is considered to be an essential trace element, necessary for growth and reproduction, and deficiency of it results in various disease conditions [31]. In mammals selenium is incorporated as selenocystein into glutathione peroxidases, the only known selenoenzyme, and into a plasma protein, called protein P, of unknown function [32]. Selenium appears also to be present in proteins as noncovalent species [32]. Although selenium is an essential nutrient a slightly (10–100-fold) elevated level is highly toxic [31,33]. How the toxicity is manifested is less well known, though it is believed that sulphydryl groups important to oxidative processes are possible sites [31].

From our results it is clear that selenite (SeO_3^{2-}), the most toxic form of selenium, has several effects on the red cell. The effect on shape is most obvious during the late stages of ATP depletion when selenite inhibits the shape transformation and stops the shape change process before completion. Selenite also appears to influence the initial stages of shape change but the effect is dependent on the history of the cells. The rate of echinocytosis of fresh cells is increased significantly in contrast to 'old' and labelled (i.e., 24 h incubated) cells that appear to be more or less unaffected. Although the interpretation is complicated by the fact that incubated 'old' cells appear to be sensitized to shape changes and thus undergo echinocytosis much quicker than fresh cells independent of whether selenite is present or not, it is clear that selenite stabilizes the cell shape during the late phase of shape transformation. Previously it has been suggested that selenite has a similar stabilizing effect during ageing on the red cell membrane fluidity and Na^+/K^+-ATPase activity [34].

It is also evident that selenite affects the metabolism of the phosphoinositides. Under ATP deplete conditions, selenite reduces the dephosphorylation of both PIP_2 and PIP whereas the turnover of PA is largely unaffected. Since this inhibition was observed when the ATP pool was nearly exhausted, it is apparent that selenite inhibits the phosphatases responsible for the degradation and not the kinases catalyzing the reverse reactions. The rise in PIP level observed at 50 µM selenite indicate not only that selenite is a strong inhibitor but also that the PIP pool is replenished by a continuos (though reduced) dephosphorylation of PIP_2. The accumulation of PIP implies also that PIP_2 is degraded to PI by phosphomonoesterases and by a route involving PIP. In addition, it is evident that selenite inhibits the phosphoinositide metabolism in a concentration-dependent manner since lower concentration of selenite caused less accumulation of PIP and less inhibition of the dephosphorylation of PIP_2.

Dephosphorylation of PIP_2 and PIP reduces the size

of their headgroups and thereby also the space they would occupy in the bilayer. Based on X-ray diffraction it has been estimated that PIP_2 occupies 0.6–0.9 nm^2 and that PI is about 0.25 nm^2 smaller than PIP_2. There are no measurements available of PIP to our knowledge but the area occupied in the membrane should be smaller than that of PIP_2 and larger than that of PI. Therefore, we have assumed that PIP is about 0.1 nm^2 smaller than PIP_2. Since most, if not all of the PIP_2 and PIP degraded are located to the inner half of the membrane, dephosphorylation would shrink the inner monolayer and thus cause a bilayer imbalance, which in turn would lead to a shape change. From geometric models the imbalance necessary to produce type 3 echinocytes (i.e., a morphological index of 3) has been calculated to 0.4–0.9% [35,36] which compares favourably with experimental estimates [36].

To transform control cells into type 3 echinocytes about 10 h depletion is necessary. During such an incubation, ~ 70% or $1.9 \cdot 10^6$ molecules of PIP_2 and ~ 25% or $0.4 \cdot 10^6$ molecules of PIP are degraded into PI. Since there are about $1.9 \cdot 10^8$ phospholipid molecules per red cell and it has been estimated that $2.7 \cdot 10^6$ (1.4%) and $1.5 \cdot 10^6$ (0.8%) of these molecules are PIP_2 and PIP, respectively, this dephosphorylation corresponds to a loss of surface area of ~ 0.54 mm^2 or 0.38% if we take the surface area of the cell to be 140 mm^2 [10]. In the presence of selenite (50 μM), ~ 55% or $1.5 \cdot 10^6$ molecules of PIP_2 are dephosphorylated during the same time but not all are converted into PI; the major fraction ($1.1 \cdot 10^6$ molecules) is converted into PIP and thereby giving rise to the increase in PIP to ~ 170% or $2.6 \cdot 10^6$ molecules. Therefore the loss in surface area in the presence of selenite is only 0.21 μm^2 or 0.15% which is apparently not enough to transform the cells into type 3 echinocytes. To obtain type 3 echinocytes, it seems that the relative contraction of the inner monolayer must be at least 0.3%. If some of the newly formed PI is translocated to the outer monolayer a larger contraction would result and these calculated bilayer imbalances should therefore be regarded as minimum values. In addition, the small fraction of PA that is converted to diacylglycerol should also contribute to the contraction of the inner monolayer, especially as diacylglycerol is believed to flip-flop rapidly [37].

That the observed shape changes both in the absence and presence of selenite coincide with the dephosphorylation of the phosphoinositides give further support to a shape controlling mechanism based on the bilayer-couple hypothesis. Although the phosphoinositides constitute only a minor fraction (< 2–4%) of total membrane lipids, the calculus indicates that the turnover of these phospholipids is sufficient, at least from a geometrical point of view, to induce shape transformation.

Red cells accumulate selenium rapidly both in vivo [38] and in vitro [39]. The red cells are also believed to have a separate mechanism for expulsion of accumulated selenium that is dependent on both the selenium concentration and GSH. From our results it is clear, in agreement with previous studies [25], that selenite is taken up by the red cell and induces a rapid consumption of reduced glutathione. It has been suggested that selenite reacts with GSH and both oxidizes it and forms a selenotrisulfide (GS-Se-SG). The selenotrisulfide is unstable and decomposes to Se and oxidized glutathione. Therefore it seems that the primary target for selenite in the red cell is GSH and that selenite causes a rapid and concentration-dependent oxidation of GSH. It is also quite likely that selenite-treatment induces oxidation of certain protein sulphydryls as selenite causes both formation of high molecular weight complexes that are dissociable upon reduction and oxidation of hemoglobin. However, it is not possible to distinguish a direct oxidative effect on protein thiols from an indirect effect caused by a drop in the GSH level.

There are several plausible ways that selenite-treatment can affect the phosphoinositide metabolism of the red cell. Selenite might directly or indirectly by its effect on the GSH level, cause oxidation of sulphydryls in the phosphatases that are important for their catalytic activities. Alternatively, the formation of high molecular weight complexes as well as hemichromes that are associated with the membrane, may simply hinder the phosphatases from accessing and hence dephosphorylating PIP_2, PIP and PA.

5. Acknowledgement

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