Oxidative Damage and Erythrocyte Membrane Transport Abnormalities in Thalassemias

By Oliviero Olivieri, Lucia De Franceschi, Maria D. Capellini, Domenico Girelli, Roberto Corrocher, and Carlo Brugnara

Oxidative damage induced by free globin chains has been implicated in the pathogenesis of the membrane abnormalities observed in α and β thalassemia. We have evaluated transport of Na+ and K+ in erythrocytes of patients with thalassemias as well as in two experimental models that use normal human red blood cells, one for α thalassemia (methylhydrazine treatment, α thalassemia like) and one for β thalassemia (phenylhydrazine treatment, β thalassemia like). With the exception of the Na-K pump, similar alterations in membrane transport were observed in thalassemia and thalassemia-like erythrocytes. These were: increased K-Cl cotransport, Na-Li countertransport and reduced Na-K-Cl cotransport. The Na-K pump was reduced in thalassemia-like cells, whereas it was increased in severe α thalassemia and in β thalassemia cells. The increased K-Cl cotransport activity could be observed in light and dense fractions of β-thalassemic cells. K-Cl cotransport in thalassemic and thalassemia-like erythrocytes was partially inhibited by [dihydro-indenyl oxy] alkanolic acid and completely abolished by dithiorthreitol. Thus, oxidative damage represents an important factor in the increased activity of the K-Cl cotransport observed in thalassemias, and of the K+ loss observed in β-thalassemia erythrocytes.

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WHEREAS MOLECULAR DEFECTS responsible for decreased globin chain synthesis in thalassemias have been described in great detail, the membrane damage imposed by the presence of free α or β chains is complex and less well characterized. Morphologic, biochemical, and metabolic changes of the erythrocyte membrane contribute to the premature destruction of thalassemic erythrocytes. Thus, membrane damage represents an important mechanism leading to anemia in thalassemias.1-4

Previous studies in thalassemic erythrocytes have shown several alterations in membrane lipids and proteins,2,4 an increase in globin binding to membrane skeleton and a decrease in membrane thiol.

One of the features that distinguishes severe β from α thalassemia is the presence of erythrocyte dehydration.5 β thalassemia minor red blood cells (RBCs) were shown to lose potassium (K+) after incubation in serum or in autologous plasma,10 probably as a consequence of the activation of the Gardos pathway induced by adenosine triphosphate (ATP) depletion and increased Ca influx.11 Studies by Nathan et al.12 suggested a relationship between this abnormal K+ leak and hemoglobin (Hb) precipitation on the membrane. Studies in sickle cell anemia, where cellular dehydration is prominent, have shown a marked activation of the K-Cl cotransport.13-15 This system induces K+ loss and cell shrinkage when the cells are swollen or exposed to acid pH.13,16 [(Dihydro-indenyl) oxy] Alkanolic acid (DIOA), a specific inhibitor for this system,16 is effective in reducing K+ loss induced by K-Cl cotransport both in normal17 and in oxygenated17 or deoxygenated sickle cells.18 K-Cl cotransport is also found in reticulocyte-rich but not in mature fractions of normal human RBCs.19 The activation of K-Cl cotransport observed in SS and CC RBCs cannot be explained exclusively on the basis of an increased number of reticulocytes. Increased K-Cl cotransport has been reported in β-thalassemia RBCs20 and in erythrocytes containing positively charged Hb variants in β6 and β7.21 Oxidation leads to activation of K-Cl cotransport in normal human RBCs21 and rabbit erythrocytes.22

Some of the features of the membrane damage observed in thalassemic RBCs can be reproduced by incorporating free α chains into normal RBCs, with the dialysis exchange hemolysis technique.23,24 An excess of denatured α or β Hb chains with associated iron, heme, or hemichromes is responsible for the generation of free radicals leading to the oxidative damage of the cell membrane in thalassemic RBCs.1,8 Schrier and Mohandas have shown that the membrane protein abnormalities observed in severe β or α thalassemia can be reproduced in normal RBCs by exposure to the oxidants phenylhydrazine (PHZ) and methylhydrazide (MHZ), respectively (β- and α-thalassemia-like cells).

In this paper, we examined the principal RBC cation transport pathways in α- and β-thalassemic RBCs. The effects on membrane transport of PHZ and MHZ treatment of normal human RBCs were also studied as well as those of DIOA and dithiothreitol (DTT) treatment.

MATERIALS AND METHODS

Drugs and chemicals. NaCl and KCl were purchased from Mallinkrodt, Inc, St Louis, MO. NaN03, albumin (bovine fraction V), TRIS(hydroxymethy1)aminomethane (TRIS), 3 (N-morpholino) propanesulfonic acid (MOPS), 2 (N-morpholino) ethanesulfonic acid, ouabain, sucrose, nystatin, bumetanide, DTT, PHZ, and MHZ were purchased from Sigma Chemical Co, St Louis, MO. MgCl2, Mg(NO3)2 and dimethylsulfoxide were purchased from Fisher Scientific Co, Fair Lawn, NJ. DIOA was purchased from Research Bio-

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Submitted October 14, 1993; accepted March 8, 1994.

Supported by Grant No. 2-P60-HL5157 from the National Institutes of Health.

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had received blood transfusions in the preceding 2 months.

**Patients.** Informed consent was obtained from control subjects and patients. Blood was drawn after overnight fasting into heparinized tubes and processed within 24 hours. Sixteen heterozygous β-thalassemic (β-thalassemia minor or β-thalassemia trait) were studied. Six were β-thalassemia patients were studied; they included two patients with Cooley’s anemia (IVS-1 n.6/IVS-1 n.6; one 9 months old, Hb 7.3 g/dL, mean corpuscular volume [MCV] 60 fl.; the other 12 years old, untransfused because of multiple RBC alloantibodies, Hb 7.5 g/dL, MCV 64 fl.), and four patients with β-thalassemia intermedia (β39/β39; Hb: 8.3, 8.5, 8.4, 9.1 g/dL; MCV 73, 70, 66 fl., respectively; two of these patients had been splenectomized). Three patients with α-thalassemia trait were studied (α-thal-1; α-thal-2; Hb: 15.1, 12.4, 10.2 g/dL; MCV: 73, 80, 82 fl., respectively). Three patients with Hb H disease (α/α-β/β-γ show 7.3, 6.0, 6.7 g/dL; MCV: 61, 62, 66 fl., respectively) were studied. None of the patients had received blood transfusions in the preceding 2 months.

**cation transport measurements.** Plasma anduffy coat were removed after centrifugation at 1200g for 100 minutes and the cells washed four times with a choline wash solution (CWS) containing 152 mmol/L choline chloride, 1 mmol/L MgCl2, 10 mmol/L TRIS-MOPS, pH 7.40 at 4°C. An aliquot of cells was then suspended in NaCl isotonic (140 mmol/L) solution containing normal Hb were incubated at 10% hematocrit with different concentrations of PHZ and MHZ (from 0.5 to 10 mmol/L in a medium containing 130 mmol/L KCl, 10 mmol/L NaCl, 1 mmol/L NaCl, 1 mmol/L MgCl2, 1 mmol/L K phosphate buffer, pH 7.4 and 10 mmol/L glucose (50 min at 37°C). At the end of the incubation, cells were washed five times in CWS and then processed as described above to measure cation transport. When experiments with DTT were performed, the oxidized cells were reincubated for additional 30 minutes at 37°C in isotonic buffered KCl (130 mmol/L) solution containing 10 mmol/L DTT. After four washes with CWS at 4°C, cells were used for K+ efflux measurement. In preliminary experiments, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed after oxidative treatment and showed membrane protein abnormalities similar to those described by Schrier and Mohandas.*

**Cell density separation.** To obtain density-fractionated light cells (density < 1.096) and dense cells (density > 1.144) discontinuous Percoll density gradients were used. Density-separated cells were washed four times with CWS at 4°C and immediately used for cation transport measurements, as described above.

**RESULTS**

**Cation transport pathways in thalassemic erythrocytes.** Hematologic data of the patients are summarized in Table 1. Activities of the major cation transport pathways in erythrocytes of β-thalassemic and α-thalassemic patients are shown in Figs 1A and 2A, respectively. To evaluate the relative magnitude of the changes in transport activities observed in thalassemic compared with normal cells, values were expressed as percentage of the control (±100% of activity). The data were also corrected for the different volume of thalassemic cells as compared with normal cells and are thus expressed based on a constant number of 1.3 × 1010 cells per liter of cells. Although quantitative differences were observed between α trait and Hb H disease, and between β trait and severe β thalassemia, qualitatively similar results were obtained for all the cation transports irrespective of the pathologic condition considered (Figs 1A and 2A). The most relevant data were (1) substantial stimulation of K-Cl cotransport and Na-Li countertransport; (2) a twofold increase of Na-K pump (with the exception of the α-trait condition); and (3) a relevant reduction of the Na-K-Cl cotransport activity (Figs 1A-2A). The Na-H exchange was not increased in either α- or β-thalassemia RBCs (Figs 1A and 2A). The different effects on four transport systems (increased Na-K pump, K-Cl cotransport, Li countertransport, and decreased Na-K-Cl cotransport) and the lack of an increase in Na-H exchange, that is known to be elevated in young cells, indicate that cell age is not the only determinant for the increased rate of transport observed in thalassemic
RBCs. Thus, studies in normal RBCs rendered thalassemia-like by exposure to PHZ or MHZ were performed to determine the effect of oxidative damage.

Cation transport properties of thalassemia-like RBCs. The mean values of the activities of the major RBC cation transport pathways in normal erythrocytes treated with different concentrations of PHZ or MHZ (β- or α-thalassemia-like erythrocytes, respectively) are shown in Figs 1B and 2B.

Exposure of erythrocytes to PHZ- and MHZ-induced changes in cation transport qualitatively similar to those of β and α thalassemia. The only noteworthy difference concerned the Na-K pump, which was strongly inhibited after exposure to both oxidant drugs (Figs 1B and 2B).

Changes in the transport activities were progressively more evident with increasing concentrations of the oxidants. For PHZ-treated erythrocytes, concentrations of 5 mmol/L yielded changes of Na-Li countertransport, Na-K-Cl and K-Cl cotransport activities similar to those of β-thalassemic cells (Fig 1B); at this concentration, Na-H exchange was

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### Table 1. Hematologic Data of Normal Controls and Thalassemic Patients

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 16)</th>
<th>β trait (n = 16)</th>
<th>β severe (n = 6)</th>
<th>α trait (n = 3)</th>
<th>Hb H (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb (g/dL)</td>
<td>13.4 ± 3.4</td>
<td>11.4 ± 2.7</td>
<td>8.2 ± 0.7</td>
<td>12.6 ± 2.5</td>
<td>8.2 ± 0.7</td>
</tr>
<tr>
<td>Hct (%)</td>
<td>45.1 ± 5.1</td>
<td>39.2 ± 4.9</td>
<td>28.5 ± 3.4</td>
<td>38.3 ± 5.3</td>
<td>33.1 ± 6.7</td>
</tr>
<tr>
<td>Cell Na (mmol/kg Hb)</td>
<td>40 ± 4.8</td>
<td>32 ± 8.1</td>
<td>23 ± 6.8</td>
<td>38 ± 7.5</td>
<td>37 ± 7.2</td>
</tr>
<tr>
<td>Cell K (mmol/kg Hb)</td>
<td>331 ± 39.3</td>
<td>265 ± 42.3</td>
<td>204 ± 26.3</td>
<td>308 ± 35</td>
<td>293 ± 27.1</td>
</tr>
<tr>
<td>Cell Na + K (mmol/kg Hb)</td>
<td>372 ± 29.3</td>
<td>286 ± 13.4</td>
<td>239 ± 15.6</td>
<td>346 ± 27.5</td>
<td>330 ± 25.6</td>
</tr>
<tr>
<td>Reticulocytes (%)</td>
<td>1.5 ± 0.3</td>
<td>5.4 ± 1.3</td>
<td>20 ± 4.7</td>
<td>2 ± 0.9</td>
<td>4.2 ± 1.4</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD.
twofold stimulated, whereas it was unaffected by lower concentrations of PHZ (Fig 1B). A progressive inhibition of the Na-K pump was observed with increasing concentrations of PHZ (Fig 1B).

For α-thalassemia-like erythrocytes, exposure to 5.7 mmol/L MHZ reproduced changes in Na-Li countertransport, Na-K and K-Cl cotransport activities, similar to those of α-thalassemic cells; at these and higher concentrations, Na-H exchange was several-fold stimulated, whereas it was unaffected by lower concentrations of MHZ (Fig 2B). Similarly to PHZ, Na-K pump was progressively inhibited by increasing concentrations of MHZ (Fig 2B).

Properties of K-Cl cotransport in thalassemic erythrocytes. In consideration of the markedly increased K loss through K-Cl cotransport observed in β-thalassemia and β-thalassemia-like RBCs (Fig 1), additional experiments were designed to elucidate the bases for the activation of the system in β thalassemia. Because K-Cl cotransport is represented mostly in young erythrocytes and reticulocytes, the observed increased activity could be a consequence of the presence of younger cells. However, if this is the case, the increased activity should still be limited to the lightest density fractions, as in normal human RBCs. K-Cl cotransport was measured in density-fractionated cells (top fraction, least-dense cells; bottom fraction, densest cells). Table 2 shows that a sizable K-Cl cotransport was present in the dense fraction of thalassemic cells, whereas it was absent in the dense fraction of normal cells. Thus, inactivation of K-Cl cotransport in cells of high density is absent in thalassemic erythrocytes. Because only three subjects with severe β-thalassemia were studied, it was not possible to determine whether splenectomy affects the activity of K-Cl cotransport in the different density fractions.

The susceptibility to inhibition by DIOA and OKA are well-recognized characteristics of K-Cl cotransport. The short-term effects of DIOA on K-Cl cotransport (measured as chloride-dependent or volume-stimulated K efflux) in α- and β-thalassemia erythrocytes are shown in Fig 3, A and B. About 50% of K-Cl cotransport was DIOA-sensitive in severe β thalassemia, Hb H disease and β-thalassemia trait, whereas in α trait, the inhibition by DIOA was only 28% (Fig 3, A and B). Similar results were obtained on both β- and α-thalassemia-like erythrocytes (PHZ = 5 mmol/L, MHZ = 5.7 mmol/L) in presence of DIOA (Fig 3) and with the protein phosphatase inhibitor OKA (10 μmol/L).

To evaluate the role of sulphhydryl (SH)-groups oxidation on K-Cl cotransport in thalassemia, K efflux was measured in thalassemia and thalassemia-like RBCs pretreated with the reducing agent DTT (10 mmol/L). DTT strongly reduced and almost normalized K+ efflux in both β- and α-thalassemia conditions (Fig 4, A and B), as well as in thalassemia-like erythrocytes (Fig 4, A and B).

DISCUSSION

The "membrane lesion" occurring in thalassemias is complex and not completely understood. Among the different changes observed, an altered cation membrane permeability has been reported, but the underlying mechanism has not been elucidated. As previously shown by others, we found that erythrocyte K+ content was significantly reduced in β-thalassemia erythrocytes (Table 1). The transport of monovalent cations was also altered in α- and β-thalassemia RBCs. We observed (1) a very strong (fourfold to ninefold) stimulation of K-Cl cotransport and Na-Li countertransport; (2) a twofold increase of Na-K pump (with the exception of the α-trait condition); (3) a relevant reduction of the Na-KCl cotransport activity and no changes in Na-H exchange activity (Figs 1A-2A). It is worth noting that K-Cl cotransport was stimulated not only in β thalassemia, but also in

| Table 2. K-Cl Cotransport in Density-Fractionated Normal Control and Thalassemic Erythrocytes |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| K-Cl Cotransport (mmol/L cell × h) | Control (n = 3) | β Trait (n = 3) | β Severe (n = 3) | α Trait (n = 3) | Hb H (n = 3) |
| Top fraction, density less than 1.096 | 2.5 ± 1.6 | 8.0 ± 2.3 | 14.6 ± 3.1 | 5.8 ± 1.7 | 6.9 ± 2.9 |
| Bottom fraction, density greater than 1.114 | 0.7 ± 0.9 | 3.0 ± 1.1 | 10.9 ± 2.1 | 3.8 ± 0.9 | 4.1 ± 1.7 |

Fig 3. Effects of DIOA and okadaic acid (OKA) on RBC K-Cl cotransport. (A) Effects on β trait, severe β-thalassemia erythrocytes, and β-thalassemia-like RBCs (PHZ, 5 mmol/L; results expressed as mean ± SD; n = 4). (B) Effects of DIOA on α trait, Hb H, and α-thalassemia-like (MHZ, 5.7 mmol/L) erythrocytes (results expressed as mean ± SD; n = 4).
CATION TRANSPORT IN THALASSEMIAS

Port, 3H-ouabain binding studies on pump activity, whereas cell age increases it. The twofold by the presence of younger cells and inhibition caused by consequence of two opposing processes: stimulation caused concomitant oxidative membrane damage. In a previous re-

inhibited in oxidized erythrocytes (Fig that in rabbit erythrocytes, oxidation decreases the Na-K pump, which was represented by the activity of the Na-K pump, which was inhibited in oxidized erythrocytes (Fig 2). It is worth noting that in rabbit erythrocytes, oxidation decreases the Na-K pump activity, whereas cell age increases it. The twofold stimulation of Na-K pump in thalassemia appears to be a consequence of two opposing processes: stimulation caused by the presence of younger cells and inhibition caused by concomitant oxidative membrane damage. In a previous report, 3H-ouabain binding studies on α- and β-thalassemia erythrocytes showed a 2.6- to 10-fold increase above normal. Because we have not measured the number of pumps in our patients, the relative effect of oxidative damage can not be determined. A discrepancy between increased pump sites and increased flux would suggest oxidative damage effect. The similarity between changes in membrane transport observed in thalassemia and thalassemia-like RBCs indicates that membrane damage rather than cell age is the main determinant of the changes we observed in thalassemia erythrocytes.

We have shown here that β-thalassemia erythrocytes have increased K-Cl cotransport (Fig 1). Another distinguishing feature of β-thalassemia erythrocytes is that increased K-Cl cotransport was observed in the top and bottom density fractions of thalassemia RBCs (Table 2). K-Cl cotransport is usually observed in the least-dense, reticulocyte-rich fraction of normal RBCs, and is absent in denser fractions. This finding is similar to previous reports in SS and CC cells and indicates that cells with increased K-Cl cotransport are present throughout the density span of thalassemia RBCs. Thus, K-Cl cotransport is a major pathway for K loss and dehydration not only in SS and CC cells, but also in β-thalassemia erythrocytes.

The role of oxidation in K-Cl cotransport activation is shown by the effects of DTT (Fig 4, A and B). Treatment with DTT reduced K-Cl cotransport in thalassemia erythrocytes to values close to control. Thus, oxidation may represen "a third factor" capable of modulating activation of K-Cl cotransport in addition to cell age and presence of β6-β7 positively charged mutations on Hb. The effect of oxidation on K-Cl cotransport has been shown in experimental models using normal cells, is poteniated by exposure to NEM and can also be shown in rabbit erythrocytes.

Membrane oxidation plays a relevant role in the alterations of membrane cation transport observed in thalassemic RBCs. It is also a major factor in the activation of K-Cl cotransport in thalassemic RBCs, which is an important determinant of the relative dehydration observed in β-thalassemia erythrocytes.

ACKNOWLEDGMENT

We thank Dr Caterina Borgna-Pignatti for providing some of the patients for this study and for helpful discussion, Drs Luciano Vetore and Concetta de Matteis for SDS-PAGE analysis, and Angela Siciliano for technical assistance.

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