Impaired Erythrocyte Methemoglobin Reduction in Sickle Cell Disease: Dependence of Methemoglobin Reduction on Reduced Nicotinamide Adenine Dinucleotide Content


We have examined aspects of methemoglobin (metHb) reduction in sickle and in thalassemic red blood cells (RBCs). NADH metHb reductase activity in sickle and thalassemic RBCs was significantly increased compared with normal RBCs. Because in vitro enzyme activity does not necessarily represent in vivo activity, we measured the rate of metHb reduction in intact RBCs. Intact thalassemic RBCs demonstrated a significantly increased rate of metHb reduction compared with normal RBCs. In contrast, intact sickle RBCs had a rate of metHb reduction that was similar to normal RBCs and significantly decreased relative to high reticulocyte RBCs of equivalent cell age. To determine the mechanism for the relative impairment of metHb reduction in sickle RBCs, we measured intracellular NADH, a cofactor in the metHb reduction reaction. Thalassemic RBCs had a significantly increased NADH content relative to normal RBCs. In contrast, sickle RBCs did not have an increase in NADH content. Furthermore, incubating normal RBCs under conditions that increase the NADH content resulted in an increased rate of metHb reduction. In contrast, conditions that decrease the NADH content in normal RBC resulted in a decreased rate of metHb reduction. These data and other results suggest that metHb reduction in intact RBCs is dependent on NADH content, and that the impaired metHb reduction rate in sickle RBCs may be a result of a lack of increase in NADH content. The dependence of metHb reduction on RBC NADH content and the ability to manipulate NADH content in vitro suggest a new strategy for decreasing oxidant damage to sickle RBCs in vivo.

Red Blood Cells (RBCs) from individuals with sickle cell disease (SCD) are more susceptible to in vivo oxidant damage than are RBC from normal individuals. RBC oxidant damage in SCD is due to the inherent instability of hemoglobin Hb S, as well as the impaired anti-oxidant defense manifested by the relative decrease in pentose phosphate shunt activity. Although it is known that Hb S is more unstable than Hb A on mechanical agitation, it was only recently that Hebbel et al. established this instability directly by demonstrating increased autooxidation of Hb S. The denaturation of Hb S is believed to occur initially through oxidation to methemoglobin (metHb) S, followed by further oxidation to hemichromes and ultimately denatured globin. 

MetHb reductases use reduced pyridine nucleotides (primarily NADH) to convert metHb to its reduced functional form. Thus, these enzymes function to interrupt the process of Hb oxidation before irreversible hemichromes are formed.

We have previously shown that RBCs from individuals with SCD have a decrease in pyridine nucleotide redox potential as manifested by a decrease in their NADH/(NAD+ + NADH) ratio. This indicates that there is a depletion of NADH relative to total NAD (NAD+ + NADH) in sickle RBCs. The latter finding, together with the lack of accumulation of metHb S in sickle RBCs, prompted us to test the hypothesis that an increased flux through NADH metHb reductase prevents metHb S accumulation and results in the depletion of NADH and the decreased NADH/(NAD+ + NADH) ratio in sickle RBCs. Thus, we examined aspects of metHb reduction in sickle RBCs. Because thalassemic RBCs are susceptible to oxidant damage, we also examined metHb reduction in thalassemia minor RBCs. In this report, we demonstrate that both sickle and thalassemic RBCs have increases in NADH metHb reductase activity. Thalassemic RBCs, which had an increased NADH content compared with normal RBCs, had an increased rate of metHb reduction in intact RBCs, whereas sickle RBCs, which had normal NADH levels, demonstrated a decreased rate of metHb reduction compared with intact thalassemic RBCs. In normal RBCs, in vitro manipulations that increase NADH content cause an increase in the rate of metHb reduction, whereas manipulations that decrease NADH content cause a decrease in the rate of metHb reduction. These results, together with other data, suggest that NADH content is a determinant of metHb reduction and that the impaired metHb reduction rate in sickle RBCs may be a result of a lack of increase in NADH content.

Materials and Methods

Procurement of blood samples. After obtaining informed consent, blood was obtained from individuals with SCD (10 patients with Hb SS, 1 patient with Hb SC, and 1 patient with Hb S-β-thalassemia) and thalassemia minor by routine venipuncture using heparin-coated tubes (15 U heparin/mL whole blood) to prevent coagulation. Blood samples from normal subjects served as controls; individuals with autoimmune hemolytic anemia and hemolytic anemia of unknown etiology were used as high reticulocyte controls. Individuals with decreased RBC glucose-6-phosphate dehydrogenase (G6PD) activity were excluded from this study. A clinical diagnosis of β-thalassemia minor was made in individuals of Mediterranean or Oriental background who had a decreased RBC mean...
corpuscular volume (MCV), RBC count greater than $5.0 \times 10^6/L$, and an increased proportion of Hb A$_2$ (determined using standard cellulose acetate electrophoresis). A clinical diagnosis of $\alpha$-thalassemia was made in individuals of Oriental background who had a decreased MCV, and increased RBC count, and a normal Hb electrophoretic pattern and normal Hb A$_2$ percentage.

Isolation of erythrocytes. An RBC-enriched fraction was prepared by passing whole blood through a column of $\alpha$-cellulose and microcrystalline cellulose to deplete white blood cells and platelets as described by Beutler. The washing of RBCs and the preparation of hemolysates have been described previously. $Hb$ was determined using the cyanmethemoglobin method.

Separation of erythrocytes on density gradients. Erythrocytes were separated by cell age using Percoll (Pharmacia LKB Biotechnology, Inc, Piscataway, NJ) and meglumine diatrizoate (ER Squibb & Sons, Inc, Princeton, NJ) density gradients as described by Vettore et al. Erythrocytes were applied onto the gradient tubes without saline washes and immediately after their passage through the cellulose column (see above). After separation, RBC fractions were washed four times with at least 20 vol of 0.15 mol/L NaCl using centrifugation.

MetHb reductase assays. MetHb reductase activity was determined in hemolysate using established spectrophotometric assays previously described by Beutler. The NADH-dependent enzyme (EC 1.6.2.2) was measured using ferricyanide as electron acceptor whereas the NADPH-dependent enzyme (EC 1.6.99.1) was measured using methylene blue as electron acceptor.

Rate of metHb reduction in intact erythrocytes. The rate of metHb reduction in intact RBCs was determined by using a slight modification of previously established methods. Only freshly obtained erythrocytes were used to determine the rate of metHb reduction; incubation of erythrocytes was begun within 2 hours of phlebotomy. To convert Hb to metHb, washed RBCs were incubated for 10 minutes at 37°C in a solution containing 0.10% (wt/vol) NaN$_3$, 6.5 mmol/L sodium phosphate, pH 7.4, and 154 mmol/L NaCl at a final packed cell volume of 25% to 30%. This resulted in 95% to 100% conversion of Hb to metHb. To remove NaNO$_3$, erythrocytes were washed a total of six times with 5 vol of 154 mmol/L NaCl using centrifugation. The incubation mixture used to measure the rate of metHb reduction contained 6.5 mmol/L sodium phosphate, pH 7.4, 154 mmol/L NaCl, 10 mmol/L D-glucose, and intact RBCs to give a final packed cell volume of 25% to 30%. The incubation mixture was maintained at 37°C. At various times during the incubation, 250-μL aliquots were withdrawn and erythrocytes were washed once with 10 vol of 154 mmol/L NaCl. The percentage of metHb remaining was then determined spectrophotometrically using the method of Hegesh et al. The rate of metHb reduction was estimated from semilogarithmic plots of metHb remaining versus time of incubation by calculating the half-life of metHb from the slope of the least-squares fit line using at least four data points. The half-life was determined in vitro for intact RBCs and for RBCs from the same individual, we examined NADH metHb reductase activity in density-separated RBCs. Hexokinase activity in RBCs from top layers was higher than in RBCs from bottom layers, suggesting that the proportion of young RBCs is higher in the top than the bottom layers (Table 1). This indicates that the density fractionation was working properly. Density separations were also examined in density-separated RBCs. Hexokinase activity in RBCs from top layers was higher than in RBCs from bottom layers, suggesting that the proportion of young RBCs is higher in the top than the bottom layers (Table 1). This indicates that the density fractionation was working properly.

 Manipulation of the NADH/[NAD$^+$ + NADH] ratio in vitro. Isolated RBCs were incubated under the same conditions used to measure the rate of metHb reduction. The NADH/[NAD$^+$ + NADH] ratio was increased by using the "pyruvate trap" method of Momsen, in which 5.0 IU of lactate dehydrogenase (LDH) and 5.0 mmol/L NADH are added to the metHb reduction incubation mixture. In this system, the presence of excess exogenous lactate oxidase drives lactate out of the cell and shifts the intracellular LDH reaction toward lactate and NAD$^+$. Other methods. The concentration of NADH in RBCs was determined using the method of Zerez et al. Hemolysate hexokinase (EC 2.7.1.1) activity was determined spectrophotometrically as described by Beutler.

Statistical analysis. All data are expressed as the mean ± the 95% confidence interval. Student's t-test was performed using standard methods.

RESULTS

NADH metHb reductase activity. RBCs from 14 normal volunteers had an NADH metHb reductase activity of 4.89 ± 0.29 IU/mL RBCs (mean ± 95% confidence interval) (Fig 1). This was not significantly different compared with RBCs from seven patients with hemolytic anemia and reticulocytosis that had an activity of 5.36 ± 1.34 IU/mL RBCs. No significant differences in NADH metHb reductase activity were found between normal whites, Orientals, and blacks (data not shown). RBCs from nine patients with SCD had an NADH metHb reductase activity of 7.62 ± 1.31 IU/mL RBCs, which was significantly increased with respect to high reticulocyte RBCs (Fig 1). RBCs from seven individuals with $\alpha$- and $\beta$-thalassemia minor had an NADH metHb reductase activity of 7.22 ± 2.12 IU/mL RBCs, which was also significantly increased with respect to normal RBCs (Fig 1). No differences in NADH metHb reductase activity were observed between $\alpha$- and $\beta$-thalassemia minor RBCs (data not shown). Thus, data from these cohorts were combined.

NADH metHb reductase activity in density-separated RBCs. To determine how metHb reductase activity varies between young and old cells from the same individual, we examined NADH metHb reductase activity in density-separated RBCs. Hexokinase activity in RBCs from top layers was higher than in RBCs from bottom layers, suggesting that the proportion of young RBCs is higher in the top than the bottom layers (Table 1). This indicates that the density fractionation was working properly. Density separations were also examined in density-separated RBCs. Hexokinase activity in RBCs from top layers was higher than in RBCs from bottom layers, suggesting that the proportion of young RBCs is higher in the top than the bottom layers (Table 1). This indicates that the density fractionation was working properly. Density separations were also examined in density-separated RBCs. Hexokinase activity in RBCs from top layers was higher than in RBCs from bottom layers, suggesting that the proportion of young RBCs is higher in the top than the bottom layers (Table 1). This indicates that the density fractionation was working properly.
rated normal and sickle RBCs demonstrated a higher NADH metHb reductase activity in cells from top layers than in cells from bottom layers (Table 1). In addition, NADH metHb reductase activity in bottom layer sickle RBCs was higher than in unfractionated normal RBC (Table 1), suggesting that the older sickle RBCs, which have a younger cell age than normal RBCs, have increased activity relative to normal unfractionated RBCs.

**Table 1. NADH metHb Reductase Activity in Hemolysates From Density-Separated RBCs From Normal Individuals and Patients With SCD**

<table>
<thead>
<tr>
<th>Enzyme Activity (IU/mL packed RBCs)</th>
<th>Unfractionated RBCs</th>
<th>Gradient-Separated RBCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hexokinase</td>
<td>0.39</td>
<td>0.61</td>
</tr>
<tr>
<td>NADH metHb reductase</td>
<td>4.06</td>
<td>6.09</td>
</tr>
<tr>
<td>SCD</td>
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<tr>
<td>Patient 1</td>
<td></td>
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</tr>
<tr>
<td>Hexokinase</td>
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<td>Patient 2</td>
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<td>1.56</td>
</tr>
<tr>
<td>NADH metHb reductase</td>
<td>6.45</td>
<td>8.64</td>
</tr>
</tbody>
</table>

**Fig 3. Rate of metHb reduction (measured as the metHb half-life) in intact RBCs from normal volunteers (NL) and from individuals with thalassemia minor (THAL), sickle cell disease (SCD), and hemolytic anemia and reticulocytosis (HR). The mean metHb half-life ± the 95% confidence interval and the range of reticulocyte percentage are shown for each of the cohorts.**

**Fig 2. Kinetics of metHb reduction.** Representative semilogarithmic plots of metHb remaining versus time of incubation are shown for intact RBCs from a normal volunteer (●), and individuals with thalassemia (□) and SCD (▲). A least-squares fit line is shown for each set of data points.

**Rate of metHb reduction.** metHb reduction closely followed an exponential decay curve; semilogarithmic plots of metHb remaining versus time of incubation typically gave straight lines (Fig 2). Therefore, we chose to express the rate of metHb reduction as a half-life. metHb half-life in intact RBCs from eight normal volunteers was 9.6 ± 1.3 hours (Fig 3). Consistent with the increased metHb reductase activity, metHb half-life was decreased to 6.5 ± 1.7 hours in RBCs from nine individuals with α- and β-thalassemia minor (Fig 3). This faster rate of metHb reduction in thalassemic RBCs was statistically significant (*P < .02*) compared with normal RBCs: metHb half-life in RBCs from 10 patients with hemolytic anemia and reticulocytosis was 6.6 ± 1.6 hours (Fig 3). However, contrary to expectation, metHb half-life in RBCs from eight patients with SCD was 9.3 ± 1.5 hours (Fig 3). The rate of metHb reduction in sickle RBCs was similar to normal RBCs, but was significantly slower compared with either thalassemic (*P < .02*) or high reticulocyte RBCs (*P < .02*). Under the same conditions used to measure metHb reduction in intact RBCs, no metHb accumulated in sickle RBCs if treatment with sodium nitrite was omitted.

**Effect of cell age on rate of metHb reduction.** metHb half-life decreased with increases in the percentage of reticulocytes (Fig 4). This suggests that the rate of metHb reduction is faster in younger RBCs. Sickle RBCs had a slower rate of metHb reduction (longer half-life) than nonsickle RBCs of equivalent cell age (Fig 4).

**Intraerythrocytic NADH content.** The relatively low rate of metHb reduction in sickle RBCs despite an increased NADH metHb reductase activity prompted an investigation of intraerythrocytic NADH levels. NADH content was similar in normal and sickle RBCs: RBCs from 15 normal volunteers and nine patients with SCD had an NADH content of 41.1 ± 3.9 and 41.6 ± 8.1 nmol/mL RBCs, respectively (Fig 5). In contrast, RBCs from 7 individuals with α- and β-thalassemia minor had an NADH content of 49.8 ± 7.9 nmol/mL RBCs (Fig 5). The increased NADH content in thalassemic RBCs was statistically significant with respect to either normal or sickle RBCs (Fig 5).

**Effect of NADH/(NAD⁺ + NADH) ratio on rate of metHb reduction.** The latter results suggested that the rate of metHb reduction in intact RBCs may be dependent on NADH content. To test this hypothesis, we examined the
The range of reticulocyte percentage are shown for each of the cohorts. (SCD). The mean NADH content IMPAIRED METHEMOGLOBIN REDUCTION IN SICKLE RBC

creases in the NADH/(NAD+ + NADH) ratio using the lactate trap technique led to a decreased rate of metHb reduction, whereas decreases in the NADH/(NAD+ + NADH) ratio resulted in decreases in the rate of metHb reduction (Fig 6, B and C).

discussion

We have examined the process of metHb reduction in sickle and thalassemic RBCs. In vitro NADH metHb reductase activity was increased significantly in both sickle and thalassemic RBCs compared with normal RBCs (Fig 1). To the best of our knowledge, this is the first published demonstration of an abnormality in NADH metHb reductase in sickle and thalassemic RBCs. This finding adds to a growing body of data that demonstrate a number of key enzyme abnormalities in thalassemia and SCD, in addition to the primary defects in Hb synthesis and structure. For example, glutathione peroxidase, superoxide dismutase, and catalase have increased activity in thalassemic RBCs. In contrast, glutathione reductase, pyrimidine 5'-nucleotidase, and phosphoribosyl pyrophosphate synthetase have decreased activity in thalassemic RBCs. Sickle RBCs are known to have an increase in the activities of superoxide dismutase and G6PD, and a decrease in the activities of catalase and glutathione reductase. Reported changes in glutathione peroxidase activity in sickle RBCs have not been consistent since this enzyme has been reported to have increased activity, decreased activity, and normal activity. Changes in Ca2+-Mg2+ ATPase activity have also not been consistent: some studies found decreased activity while other studies found increased activity.

In contrast to the increased NADH metHb reductase activity, no significant differences were found in NADPH metHb reductase (ie, NADPH diaphorase) between normal, sickle, and thalassemic RBCs. metHb reduction occurs via the NADH and the NADPH-dependent reductase. However, in the intact RBCs, the NADH-dependent enzyme is responsible for most of the metHb reduction whereas the NADPH-dependent enzyme plays a minor role.

Erythrocytes from patients with SCD have been shown to have heterogeneous densities with an increase in the proportion of both high- and low-density RBCs. Thus, the increase in NADH metHb reductase in unfractionated sickle RBCs may be due to a disproportionate increase in either high- or low-density RBCs. This prompted an examination of NADH metHb reductase activity in density-separated sickle RBCs. We found that both high- and low-density sickle RBCs had an increase in NADH metHb reductase activity (Table 1), suggesting that the increase in enzyme activity in unfractionated sickle RBCs is due to an increase in activity in both high- and low-density RBCs. In addition, low-density RBCs from both normal individuals and sickle patients had higher NADH metHb reductase activity than their high-density RBC counterparts, suggesting that younger RBCs have more enzyme activity than older RBCs.

We examined the capacity of intact RBCs to reduce metHb because the increase in NADH metHb reductase activity in vitro does not necessarily indicate an increase in activity in vivo. In addition, our use of the more functional intact RBC assay for metHb reduction may resolve the apparent paradox of increased free heme content, which implies faster Hb S denaturation, together with increased NADH metHb reductase activity in sickle RBCs. Using the more functional assay for metHb reduction, intact thalassemic RBCs had a faster rate of metHb reduction (shorter metHb half-life), which was consistent with the increase in NADH metHb reductase activity. However, intact sickle RBCs had a metHb reduction rate that was decreased (longer metHb half-life) relative to RBCs of equivalent cell age (Figs 3 and 4) and relative to thalassemic RBCs (Fig 3). This suggested...
that sickle RBCs contain factors that prevent an increase in the rate of metHb reduction despite the increase in NADH metHb reductase activity. Our observation that sickle RBCs do not undergo spontaneous metHb formation under the conditions that we used suggests that once metHb S is formed it is either quickly reduced back to Hb S or oxidized further to hemichrome S and then to denatured globin S after heme release. This is consistent with the quick heme loss (and therefore further denaturation) once metHb S is formed,\(^1\) and with the increase in membrane\(^6\) and cytoplasm\(^6\) free heme content in sickle RBCs.

A number of mechanisms could be responsible for the relative impairment in the rate of metHb reduction in sickle RBCs. One possibility is that NAD\(^+\) content, which is elevated approximately twofold in sickle RBCs,\(^4\) may be inhibiting NADH metHb reductase activity in vivo (such a mechanism would not cause a decrease in activity in vitro because the hemolysate, and therefore the endogenous NAD\(^+\), is diluted out before assay). We found that NAD\(^+\) had no significant effect on NADH metHb reductase activity even when present at 350 \(\mu\)mol/L, a concentration that is four times higher than that found in sickle RBCs (data not shown). This indicates that impaired metHb reduction is not due to the increased NAD\(^+\) content of sickle RBCs. The other possibility is that sickle RBCs have a decreased NADH content. Although NADH content in sickle RBCs was similar to that of normal RBCs, it was significantly decreased relative to thalassemic RBCs (Fig 5). This suggests that NADH content may be a determinant of metHb reduction in intact RBCs.

Previous data from this laboratory\(^1\) show that NADH content is not significantly increased in high reticulocyte RBC compared with sickle RBCs. Although measured intracellular NADH concentrations reflect the sum of both bound and free NADH,\(^6\) it is the free NADH concentration that is important in regulating the rate of enzyme reactions in which NADH is a cofactor.\(^6\) Thus, our inability to demonstrate a significant increase in the NADH content of high reticulocyte RBCs compared with sickle RBCs does not necessarily contradict the hypothesis that NADH content is a determinant of metHb reduction.

To provide a more functional test of the hypothesis that metHb reduction is dependent on NADH content, we examined the effect of manipulating the NADH/(NAD\(^+\) + NADH) ratio on the rate of metHb reduction in normal RBCs. If NADH is a determinant of metHb reduction, then increasing the NADH/(NAD\(^+\) + NADH) ratio with the pyruvate trap technique would increase the rate of metHb reduction, and decreasing the NADH/(NAD\(^+\) + NADH) ratio with the lactate trap technique would decrease the rate of metHb reduction. This is precisely the pattern that we found when normal RBCs were used (Fig 6A). More importantly, the same pattern was obtained with thalassemic (Fig 6B) and sickle RBCs (Fig 6C). As expected, the use of...
the pyruvate trap to increase the NADH/(NAD\(^+\) + NADH) ratio in sickle RBCs resulted in rates of metHb reduction that were similar to those in untreatedthalassemic RBCs (Figs 3 and 6C). This demonstrates that it is possible toamplify the impaired metHb reduction rate in sickle RBCs by increasing the NADH/(NAD\(^+\) + NADH) ratio. These findings provide further support for the hypothesis that NADH is a determinant of metHb reduction in normal as well as thalassemic and sickle RBCs. Furthermore, the increase in metHb reduction rate in sickle RBCs in response to an increase in the NADH/(NAD\(^+\) + NADH) ratio excludes the possibility that the impairment in metHb reduction in sickle RBCs is due to the inability of metHb S to undergo reduction. However, our data do not exclude the possibility that impaired metHb reduction is due, in part, to a smaller rate of reduction of metHb S by the metHb reductase in sickle RBCs.

Because we have shown that NADH is a determinant of metHb reduction in intact RBCs and that impaired metHb reduction in sickle RBCs may be the result of a lack of increase in NADH content, the hypothesis that the decreased NADH/(NAD\(^+\) + NADH) ratio in sickle RBCs is a result of Hb S instability
d and hence increased NADH consumption is no longer tenable. Thus, the mechanism for the decreased NADH/(NAD\(^+\) + NADH) ratio in sickle RBCs remains to be determined.

The dependence of metHb reduction on NADH content underscores the important role that pyridine nucleotides play in RBC oxidant damage and its prevention. The lack of increase in NADH content in sickle RBCs and the resulting impaired ability to reduce metHb represent a novel mechanism to explain the oxidant sensitivity of sickle RBCs. More importantly, the ability to manipulate the intraerythrocytic NADH content in vitro suggests a new strategy for decreasing oxidant damage to sickle RBCs and perhaps for the treatment of SCD.

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Impaired erythrocyte methemoglobin reduction in sickle cell disease: dependence of methemoglobin reduction on reduced nicotinamide adenine dinucleotide content

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