CONCISE REPORT

Decreased Erythrocyte Nicotinamide Adenine Dinucleotide Redox Potential and Abnormal Pyridine Nucleotide Content in Sickle Cell Disease


RBCs from individuals with sickle cell disease are more susceptible to oxidant damage. Because key antioxidant defense reactions are linked to the pyridine nucleotides nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP), we tested the hypothesis that the RBC redox potential as manifested by the NADH/[NADH + NADH] and NADPH/[NADP + NADPH] ratios is decreased in sickle erythrocytes. Our data demonstrate that sickle RBCs have a significant decrease in the NADH/[NADH + NADH] ratio compared with normal RBCs (P < .00005). Interestingly, sickle RBCs also had a significant increase in total NAD content compared with normal RBCs (P < .0005). In contrast, although sickle RBCs had a significant increase in the total NADP content compared with normal RBCs (P < .00005), sickle RBCs had no significant alteration in the NADPH/[NADP + NADPH] ratio. High reticulocyte counts demonstrated that these changes were not related to cell age. Thus, sickle RBCs have a decrease in NAD redox potential that may be a reflection of their increased oxidant sensitivity. The changes in these pyridine nucleotides may have further metabolic consequences for the sickle erythrocyte.

Denaturation of Hb S is believed to occur through oxidation to methemoglobin (met-Hb) with the subsequent formation of hemichromes and ultimately denatured globin. Methemoglobin reductases interrupt this sequence and constitute the first defense mechanism to reverse this process. These reductases use reduced nicotinamide adenine dinucleotide (NADH) and, to a limited extent, reduced nicotinamide adenine dinucleotide phosphate (NADPH) to convert met-Hb back to its more stable, reduced form. Because Hb S is more unstable than Hb A, Hb S may be oxidized to met-Hb S more readily than Hb A to met-Hb A. Although this possibility has not been demonstrated directly, it has recently been suggested as a cause of Hb S instability by Hebel. Thus, we tested the hypothesis that sickle RBC have a decrease in NADH relative to the total NAD (NADH + NADPH) concentration. The data presented in this report indicate that sickle RBC have a decrease in their NADH/NADPH ratio and an increase in their NAD1 and NADP1 concentrations.

MATERIALS AND METHODS

Blood samples. After obtaining informed consent, blood was obtained by routine venipuncture with heparin (15 U/mL whole blood) as an anticoagulant. Individuals with SCD were adult homozygotes (Hb SS) who did not have concomitant RBC G6PD deficiency. Normal subjects and individuals with reticulocytosis were used as controls. None of the subjects received a blood transfusion within the 3 months before the study.

Determination of pyridine nucleotides. The concentrations of the pyridine nucleotides in RBC were determined by using the method of Zerez et al. Twenty microliters of blood were mixed with 1,980 µL of a solution containing 10 mmol/L nicotinamide, 20 mmol/L NaHCO3, and 100 mmol/L Na2CO3 at 0°C. The mixture was frozen in a dry ice-acetone bath, thawed quickly in a room temperature water bath, and then chilled to 0°C. To destroy NAD+ and NADP+, 700 µL of this mixture was incubated at 60°C for 30 minutes and promptly chilled to 0°C. Both the heat-treated extract,
which contained only NADH and NADPH, and the untreated extract, which contained NADH, NAD\(^+\), NADPH, and NADP\(^+\), were immediately analyzed by using spectrophotometric cycling assays.\(^{15}\) The concentrations of NAD\(^+\) and NADP\(^+\) were obtained by subtracting the concentration of NADH or NADPH in the heated extract from the concentration of NAD\(_T\) or NADP\(_T\) in the unheated extract.

**RESULTS**

Sickle RBC (8\% to 54\% reticulocytes) had a significant increase in the NAD\(_T\) concentration compared with normal RBC and with RBC from individuals with a moderately (2.0\% to 6.5\%, HR I) or a markedly (18.7\% to 76.0\%, HR II) increased reticulocyte count (Fig 1A). The NADH concentration (mean ± 1 SD) in normal, HR I, HR II, and SCD RBC was 42.2 ± 8.3, 43.0 ± 10.6, 46.7 ± 8.8, and 41.6 ± 10.5 nmol/mL packed RBC, respectively. Thus, the RBC NADH content appeared to be remarkably constant in all cohorts examined. The NAD\(^+\) concentration, however, was significantly increased in sickle RBC compared with normal, HR I, or HR II RBC (Fig 1B), which indicates that the increase in NAD\(_T\) content in sickle RBC is due entirely to the increase in NAD\(^+\) content. Thus, the ratio of NADH/NAD\(_T\), which is an indicator of cellular redox potential,\(^{16}\) was decreased significantly in sickle RBC compared with either normal, HR I, or HR II RBC (Fig 1C).

Sickle RBC had a significant increase in the NADP\(_T\) concentration compared with normal, HR I, or HR II RBC (Table 1). In addition, the NADPH content was also increased significantly in sickle RBC compared with normal, HR I, or HR II RBC (Table 1). However, no significant differences were found in the NADPH/NADP\(_T\) ratio between SCD, normal, HR I, and HR II RBC (Table 1). In contrast to the increase in NAD\(_T\) content, the increase in NADP\(_T\) content in sickle RBC could be accounted for only by the measurable increase in NADPH content, given that the increase in NADP\(^+\) content was not statistically significant (Table 1). The unchanged NADPH/NADP\(_T\) ratio coupled with the increased NADPH content suggest that there should be a concomitant increase in NADP\(^+\) content in sickle RBC. We were not able to measure such an increase because RBC NADP\(^+\) is present in trace amounts that are difficult to quantitate accurately.\(^{17}\)

We next sought to exclude potential sources of error for our observations. No differences were observed in RBC
and NADP were completely destroyed and added NADH was obtained in unheated extracts, whereas added NAD, NADH, NADP, and NADPH were recovered with yields ranging between 91% and 100%. Thus, the recovery of added pyridine nucleotides during the extraction process.

In heated extracts of sickle RBC, added NAD and NADP were recovered with yields ranging between 91% and 100%. Thus, the recovery of added pyridine nucleotides was identical in normal, high reticulocyte, and SCD blood samples. To ensure full recovery of pyridine nucleotides during extraction of sickle RBC, we repeated this recovery experiment by using blood samples from patients with SCD. In unheated extracts of sickle RBC, added NAD+, NADH, NADP+, and NADPH were recovered with yields ranging between 96% and 100%. In heated extracts of sickle RBC, added NAD+ and NADP+ were destroyed completely, whereas added NADH and NADPH were recovered with yields ranging between 91% and 100%. Thus, the recovery of added pyridine nucleotides was identical in normal, high reticulocyte, and SCD blood samples. This suggests that the abnormalities in pyridine nucleotide content in sickle RBC were not due to differences in recovery of the nucleotides during the extraction process. In addition, because hemoglobin can mediate the oxidation of both NADPH and NADH during extraction of RBC under alkaline conditions, the decrease in the NAD+/NADH ratio compared with the unchaged NAD+/NADP+ ratio in sickle RBC provides additional evidence that our data are not due to an artifact of the pyridine nucleotide extraction or assay systems.

We as well as other investigators have used whole blood to determine the RBC pyridine nucleotide content because previous studies have shown that RBC have at least a 100-fold higher concentration of pyridine nucleotides than do leukocytes. Another possible contributor to "RBC" pyridine nucleotide content when whole blood is used, however, is plasma. Thus, although unlikely, the abnormal pyridine nucleotide content in sickle whole blood might have been due to changes in the plasma pyridine nucleotide content. We found no differences in NAD+ or NADP+ contents between normal and sickle plasma (data not shown). More importantly, plasma NAD+ and NADP+ were both found at concentrations less than 1 nmol/mL plasma, which indicates that they make an insignificant contribution to the whole blood pyridine nucleotide content. These results suggest that the changes in pyridine nucleotide content in whole sickle blood reflect changes in RBC pyridine nucleotide content.

**DISCUSSION**

We have demonstrated that sickle RBC have an abnormal pyridine nucleotide content as manifested by a decrease in the NADH/NAD+ ratio and by increases in the NAD+, NADH, NADP+, and NADPH contents. It is interesting that although the increase in NAD+ was accounted for entirely by an increase in NAD+, the increase in NADP+ was accounted for by an increase in NADPH. Although the high reticulocyte RBC control groups did have a slight decrease in the NADH/NAD+ ratio and slight increases in NAD+ and NADP+ contents compared with normal RBC, sickle RBC had a greater decrease in the NADH/NAD+ ratio and greater increases in NAD+ and NADP+ contents compared with normal RBC. These results suggest that the pyridine nucleotide abnormalities found in sickle RBC are not a reflection of a younger mean RBC age. The finding of statistical significance in RBC pyridine nucleotide content and ratios between HR II and SCD confirms the latter hypothesis.

The increases in NAD+ and NADPH contents may cause additional and perhaps adverse metabolic consequences in sickle RBC. For example, because NADPH is a potent inhibitor of G6PD, the increased NADPH content is a possible mechanism for the relative impairment in pentose phosphate shunt activity in sickle RBC. Furthermore, because NAD+ is a rate-limiting factor in the glyceraldehyde-3-phosphate dehydrogenase reaction, the increased NAD+ content is a possible mechanism for the increased glucose consumption rate of sickle RBC (Lachant, Zerez and Tanaka, unpublished results).

The decrease in the NADH/NAD+ ratio suggests that sickle RBC have a decrease in the NAD redox potential. In

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**Table 1. NADP Concentrations and NADPH/NADP+ Ratio in RBC From Normal Subjects, Individuals With SCD and Individuals With Reticulocytosis**

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Reticulocytes (%)</th>
<th>Pyridine Nucleotides (nmol/mL Packed RBC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (n = 16)</td>
<td>Mean ± 1 SD</td>
<td>NADP</td>
</tr>
<tr>
<td>Mean ± 1 SD</td>
<td>0.9 ± 0.4</td>
<td>39.5 ± 3.7</td>
</tr>
<tr>
<td>Range</td>
<td>0.5-1.1</td>
<td>33.6-48.2</td>
</tr>
<tr>
<td>HR I (n = 8)</td>
<td>Mean ± 1 SD</td>
<td>3.3 ± 0.9</td>
</tr>
<tr>
<td>Range</td>
<td>2.0-5.0</td>
<td>36.2-64.5</td>
</tr>
<tr>
<td>HR II (n = 5)</td>
<td>Mean ± 1 SD</td>
<td>32.9 ± 26.2</td>
</tr>
<tr>
<td>Range</td>
<td>11.3-76.0</td>
<td>37.9-56.9</td>
</tr>
<tr>
<td>SCD (n = 9)</td>
<td>Mean ± 1 SD</td>
<td>28.2 ± 15.4</td>
</tr>
<tr>
<td>Range</td>
<td>8.1-53.7</td>
<td>44.4-76.9</td>
</tr>
</tbody>
</table>

T-test: NADP+: normal v SCD, P < .00005; HR I v SCD, P < .02; HR II v SCD, P < .05. NADPH: normal v SCD, P < .00005; HR I v SCD, P < .02; HR II v SCD, P < .05. NADPH/NADP+: normal v SCD, HR I, and HR II, not significant. NADPH/NADP+: SCD v normal, HR I, and HR II, not significant.
contrast, we observed no apparent changes in the NADPH/NADP⁺ ratio in sickle RBC. This is consistent with the minor role that NADPH methemoglobin reductase is believed to play in the RBC.13 Our results suggest that the decrease in NAD redox potential may be a manifestation of increased oxidant sensitivity and increased Hb S instability in sickle RBC. Studies are currently under way in our laboratory to determine the mechanism for the increases in NAD⁺ and NADP⁺ contents in sickle RBC.

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REFERENCES

Decreased erythrocyte nicotinamide adenine dinucleotide redox potential and abnormal pyridine nucleotide content in sickle cell disease

CR Zerez, NA Lachant, SJ Lee and KR Tanaka