CONCISE REPORT

Abnormal Erythrocyte Calcium Homeostasis in Oxidant-Induced Hemolytic Disease

By Oded Shalev, Mary N. Leida, Robert P. Hebbel, Harry S. Jacob, and John W. Eaton

The processes leading to red cell destruction in oxidant-induced hemolytic disease are not yet fully known. Oxidant damage to hemoglobin per se may be insufficient to explain the process, and the involvement of membrane damage has been suggested. We now report that at least one crucial membrane function—exclusion of calcium—is disrupted by the potent oxidant phenylhydrazine. Phenylhydrazine has long been employed in the experimental production of Heinz body hemolytic disease and, historically, has been used as therapy for polycythemia vera. Reactions between phenylhydrazine and hemoglobin are known to generate both superoxide and hydrogen peroxide. These species of activated oxygen and others, such as hydroxyl radical, may then damage various cellular constituents. Perhaps because the most obvious products are oxidized forms of hemoglobin—methemoglobin and sulfhemoglobin—attention has been focused on this process. Recently, however, the involvement of membrane damage in oxidant hemolysis has been suggested.

Although many membrane components are possible targets for oxidants, one in particular—calcium ATPase—may be of crucial importance for the survival of red cells. Calcium ATPase contains one or more reactive-SH groups, oxidation of which leads to complete inhibition of enzyme activity. This enzyme normally functions to maintain the very steep gradient between external (~10^{-3} M) and intracellular (~10^{-8} M) calcium, and collapse of this gradient is associated with decreased red cell deformability and premature destruction. Indeed, the accumulation of calcium triggered by exposure of nucleated cells to a variety of toxins has been suggested as a final common pathway of cell death.

In view of the above, we hypothesized that oxidants such as phenylhydrazine might inactivate erythrocyte calcium ATPase. Such inhibition would predispose the cell to accumulation of calcium. The resultant elevation of intraerythrocytic calcium might then be important in the genesis of oxidant-induced hemolytic disease.

MATERIALS AND METHODS

In Vitro Studies

Normal human red cells from fresh heparinized blood were washed 3 times with isotonic saline, the buffy coat being aspirated after each wash. The RBCs were incubated at 37°C for 30 min at 10% hematocrit (Hct) in Hank's balanced salt solution (pH 7.4) containing concentrations of phenylhydrazine ranging from 0.05 to 10 mM. After incubation, the cells were washed 3 times in isotonic Tris-HCl (0.172 M, pH 7.6) and assayed for Ca^{2+} ATPase activity. Briefly, the washed RBCs were brought to Hct 10% with the Tris-HCl buffer and isotonic lysis was induced by saponin. The Ca^{2+} ATPase assay mixture contained 0.1 ml of lystate, 0.2 ml of the Tris-HCl buffer, and 0.7 ml of a reaction mixture containing 30 mM imidazole, 100 mM KCl, 15 mM NaH2PO4, 4.5 mM MgCl2, 300 μM ouabain, and 4.5 mM ATP. Finally, 0.02 ml of either 1.25 mM CaCl2 or 5 mM EGTA was added, the final concentrations being 25 μM and 100 μM, respectively. The total volume of the assay mixture was 1.02 ml. Following precipitation with 0.1 ml of 50% trichloroacetic acid (TCA), inorganic phosphorous (P_i) in the supernatant was measured using the method of Fiske-Subbarow. Calcium ATPase activity was calculated by subtracting the P_i content of the EGTA-containing sample (Mg^{2+}-ATPase) from that of the calcium-containing sample (Ca^{2+}-Mg^{2+}-ATPase). Results were expressed as μmole P_{i} liberated/g Hb/hr at 37°C.

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Supported in part by NIH Grants HL-16833 and HL-26139.

J.W.E. is recipient of an NIH Career Development Award.

Submitted July 13, 1981; accepted July 29, 1981.

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* 1981 by Grune & Stratton, Inc. 0006-4971/81/5806-0027$01.00/0

Blood, Vol. 58, No. 6 (December), 1981
activity was seen when the concentration was increased to 10 mM. Encouraged by these results, we examined the in vivo effect on mouse RBC Ca\(^{++}\) ATPase and intracellular calcium content (Fig. 2). One hour after phenylhydrazine injection, Ca\(^{++}\) ATPase activity was markedly inhibited, declining from a mean of 69 \(\mu\)mole/g Hb/hr to a mean of 4 \(\mu\)mole/g Hb/hr. Even 24 hr after injection of phenylhydrazine, Ca\(^{++}\) ATPase activity remained below 20% of normal values. Substantial recovery in the enzyme activity was noted within 48 hr, approaching 80% of baseline values. Nevertheless, even after 7 days, and despite reticulocytosis of 25%, the enzyme activity still had not returned to normal.

Coincident with this inhibition of Ca\(^{++}\) ATPase activity, RBC Ca content increased markedly. At 1 hr postinjection, RBC Ca content was elevated from 7.5 \(\mu\)M/liter RBC to a mean value of 32.8 \(\mu\)M/liter RBC. At 24, 48 hr, and 7 days, the mean values were 22.5, 29.9, and 17.6, respectively. All postphenylhydrazine RBC Ca levels were significantly higher than those of control animals. The results are summarized in Table I, together with the phenylhydrazine-induced changes in Hb, Hct, and reticulocyte count.

RESULTS

Initially, the effect of varying concentrations of phenylhydrazine on the activity of human RBC membrane Ca\(^{++}\) ATPase was studied. Whereas at 0.1 mM there was minimal change in enzyme activity, a 1 mM concentration inhibited the enzyme by 40%–45% (Fig. 1). More pronounced inhibition of the enzyme activity (~75%) was seen when the concentration was increased to 10 mM. Encouraged by these results, we examined the in vivo effect on mouse RBC Ca\(^{++}\) ATPase and intracellular calcium content (Fig. 2). One hour after phenylhydrazine injection, Ca\(^{++}\) ATPase activity was markedly inhibited, declining from a mean of 69 \(\mu\)mole/g Hb/hr to a mean of 4 \(\mu\)mole/g Hb/hr. Even 24 hr after injection of phenylhydrazine, Ca\(^{++}\) ATPase activity remained below 20% of normal values. Substantial recovery in the enzyme activity was noted within 48 hr, approaching 80% of baseline values. Nevertheless, even after 7 days, and despite reticulocytosis of 25%, the enzyme activity still had not returned to normal.

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DISCUSSION

The precise nature of the cellular lesions responsible for oxidant-induced hemolytic disease is not known. It
is clear that the RBC oxidant damage that occurs in certain congenital red cell enzyme defects such as glucose-6-phosphate dehydrogenase deficiency, as well as that caused by phenylhydrazine administration, leads to oxidation of intracellular hemoglobin and the formation of Heinz bodies. It is also evident that cells containing Heinz bodies are at risk for splenic sequestration and destruction. Although the effects of oxidants on intracellular hemoglobin may be the most readily observed phenomenon in oxidant-induced hemolytic disease, damage to other cellular constituents also occurs. Moreover, Johnson et al. have recently suggested that oxidation of membrane sulfhydryl groups may lead to deleterious changes in both the deformability and survival of red cells.

In keeping with this possibility, it has been found that Ca\(^{++}\) ATPase, a crucial membrane enzyme, is readily inhibited by a variety of sulfhydryl reagents. Normal functioning of this enzyme is important in the maintenance of the normal (and very low) intr erythrocytic calcium content. Substantial inhibition of this enzyme may lead to calcium accumulation, which, in turn, may result in abnormal membrane deformability and accelerated red cell destruction. In the present experiments, we have found that the potent oxidant, phenylhydrazine, causes inhibition of human RBC Ca\(^{++}\) ATPase during in vitro incubations. Even more striking inhibition of this enzyme was observed following administration of a small dose of phenylhydrazine to mice. This inhibition of enzyme activity was perceptible up to 1 wk after phenylhydrazine injection, suggesting that the damage to the enzyme is, at least to a certain extent, irreversible. Accompanying this profound inhibition of Ca\(^{++}\) ATPase activity, RBC Ca\(^{++}\) content rose to values 3-4 times normal shortly after phenylhydrazine treatment. These increased RBC Ca\(^{++}\) levels persisted for at least 2 days.

Thus, at least one oxidant drug—phenylhydrazine—causes serious disturbances in RBC Ca\(^{++}\) balance. Administration of this agent leads to profound inhibition of mouse red cell Ca\(^{++}\) ATPase activity and a coordinate rise in cell calcium content. Any substantial increase in RBC Ca\(^{++}\) is likely to affect adversely the circulation and survival of the cell. Therefore, oxidant-induced impairment of Ca\(^{++}\) homeostasis may well contribute to the genesis of the hemolytic process. The importance of this phenomenon in oxidant hemolytic disease caused by other drugs and by enzyme deficiencies awaits elucidation.

ACKNOWLEDGMENT

The authors thank Betsy Nelson for technical assistance and Diane Konzen for help in preparation of this manuscript.

REFERENCES


Table 1. Mean and Range of RBC Ca\(^{++}\) ATPase Activity, RBC Ca\(^{++}\) Content, Hemoglobin, Hematocrit, and Reticulocyte Count in Phenylhydrazine-Injected Mice

<table>
<thead>
<tr>
<th>Time Post Injection</th>
<th>RBC Ca(^{++}) ATPase (μmole/Pg Hb/hr)</th>
<th>RBC (Ca) (μmole/liter)</th>
<th>Hemoglobin (g/dl)</th>
<th>Hematocrit (%)</th>
<th>Reticulocytes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, mean</td>
<td>69</td>
<td>7.5</td>
<td>14.3</td>
<td>41</td>
<td>1.5</td>
</tr>
<tr>
<td>Range</td>
<td>68-70</td>
<td>5.2-7.8</td>
<td>13.6-15</td>
<td>40-42</td>
<td>1-2</td>
</tr>
<tr>
<td>1 hr, mean</td>
<td>4</td>
<td>32.3</td>
<td>40.4</td>
<td>35</td>
<td>1.5</td>
</tr>
<tr>
<td>Range</td>
<td>3-5</td>
<td>31.1-34.5</td>
<td>10.2-10.6</td>
<td>34.6-35.3</td>
<td>1-2</td>
</tr>
<tr>
<td>24 hr, mean</td>
<td>13</td>
<td>22.5</td>
<td>9.8</td>
<td>33.8</td>
<td>12</td>
</tr>
<tr>
<td>Range</td>
<td>10-16</td>
<td>17.3-27.7</td>
<td>9.6-10</td>
<td>33.1-34.5</td>
<td>10-14</td>
</tr>
<tr>
<td>48 hr, mean</td>
<td>54</td>
<td>29.9</td>
<td>10</td>
<td>32.7</td>
<td>18</td>
</tr>
<tr>
<td>Range</td>
<td>48-62</td>
<td>21.4-38.5</td>
<td>8.7-11.3</td>
<td>32.2-33.2</td>
<td>15-20</td>
</tr>
<tr>
<td>7 days, mean</td>
<td>58</td>
<td>17.6</td>
<td>11.4</td>
<td>36.5</td>
<td>27</td>
</tr>
<tr>
<td>Range</td>
<td>55-61</td>
<td>15.9-19.3</td>
<td>11-11.7</td>
<td>36-37</td>
<td>25-28</td>
</tr>
</tbody>
</table>

(t = 91.7, p < 0.001) (t = 19.8, p < 0.001)
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