Catalase and Glutathione Peroxidase Are Equally Active in Detoxification of Hydrogen Peroxide in Human Erythrocytes

By Gian Franco Gaetani, Silvana Galiano, Letizia Canepa, Anna Maria Ferraris, and Henry Neil Kirkman

Although human erythrocytes contain large amounts of catalase (H$_2$O$_2$; H$_2$O$_2$ oxidoreductase, EC 1.11.1.6), the principal means for disposal of hydrogen peroxide in these cells has been considered to be the NADPH, glutathione reductase/peroxidase pathway. Supporting this traditional viewpoint has been the susceptibility to peroxidative stress of cells with impaired production of NADPH. Recent studies, however, revealed that each stress of cells with impaired production of NADPH/glutathione/glutathione peroxidase pathway is the principal means for disposal of H$_2$O$_2$ in human erythrocytes. Recently, however, mammalian catalase was found to have tightly bound NADPH and to require NADPH for the prevention and reversal of inactivation by its toxic substrate (H$_2$O$_2$). Since both catalase and the glutathione pathway are dependent on NADPH for function, this finding raises the possibility that both mechanisms destroy H$_2$O$_2$ in human erythrocytes. A comparison of normal and acatalasemic erythrocytes in the present study indicated that catalase accounts for more than half of the destruction of H$_2$O$_2$ when H$_2$O$_2$ is generated at a rate comparable to that which leads to hemolysis in G6PD-deficient erythrocytes.

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MATERIALS AND METHODS

Samples of blood were obtained from five healthy men, one acatalasemic man, and four G6PD-deficient men carrying the Mediterranean variant.

Collection of CO$_2$. Leukocytes and platelets were removed by filtration of the heparinized blood through cellulose. Plasma was removed, and the erythrocytes were washed by dilution in 5 vol 0.15 mol/L NaCl and centrifugation and then suspended in Krebs-Ringer solution/N-Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (Tes) buffer, pH 7.4, containing glucose at a final concentration of 5 mmol/L. For studies of hexose monophosphate shunt (HMS) and glutathione, suspensions of packed erythrocytes were mixed with an equal volume of Krebs-Ringer solution/Tes buffer (pH 7.4)/5 mmol/L glucose (KRTG). The packed cell volume ranged between 45% and 50% of total volume. Incubation mixtures comprised 1.0 mL erythrocyte suspension, 1.0 mL KRTG solution (with or without glucose oxidase), and 0.3 mL (0.5 μCi) of [1-¹⁴C] glucose or [2-¹⁴C] glucose (specific activity 3 μCi/μmol; Amersham Int, Buckinghamshire, England). All incubations were performed in duplicate. For each incubation, the HMS activity is represented by the sum of the mean of the radioactivity evolved from the separate incubations with [1-¹⁴C] and [2-¹⁴C] glucoses. For assays of reduced glutathione (GSH), labeled glucoses were omitted. For studies of HMS, suspensions of erythrocytes were incubated in 25-mL vials to which were attached rubber caps containing disposable center wells (Kontes, Vineland, NJ). Throughout the one-hour incubation, the center wells contained 0.2 mL 1 N NaOH. At the end of the incubation, 0.7 mL 3.7 mol/L perchloric acid was injected by needle through the rubber cap into the incubation mixture.
described by Beutler et al. Glucose concentration was measured by form 14C-glucose. The containing H2O2 DETOXIFICATION IN HUMAN ERTHROCYTES glucose oxidase were carried out under conditions of labeled glucose at zero time and the total amount of glucose and hexokinase. The glucose concentration, together with measurement of 14CO2, allowed calculation of the specific activity of labeled glucose at zero time and the total amount of glucose consumed over 60 minutes.

Measurement of rate of generation of H2O2. Addition of glucose oxidase to the incubation mixtures served to expose the erythrocytes to steady-state concentrations of H2O2, as described by Chance and Aebi and Suter. Preliminary assays of glucose oxidase permitted a choice to be made of the concentration of glucose oxidase that would allow continuous generation of H2O2 at the desired rate. Assays for glucose oxidase were carried out under conditions identical to those for incubation of erythrocytes: Variable amounts of glucose oxidase were added to 2.3 mL Krebs-Ringer/Tes buffer, pH 7.4, to a final enzyme concentration of 2.2, 4.5, and 9.0 nmol/L. The reaction was followed at 37°C in a 3.0-mL cuvette with a light path of 1 cm. In addition, the assay mixture contained horseradish peroxidase, phenol, and 4-aminoantipyrine as specified for the spectrophotometric determination of H2O2 by the method of Green and Hill. Under these conditions, the rate of generation of H2O2 was constant for one hour at concentrations of glucose oxidase up to 9 nmol/L.

Catalase and NADH. Sephacryl S-200 was a product of Pharmacia Fine Chemicals. Ultrafiltration was performed with CF-25 ultrafiltration cones from Amicon in the manner described earlier. Protein concentrations were measured by the Folin method. Catalase activity was expressed as the first-order kinetic constant of the rate of disappearance of H2O2 as measured by absorbance at 240 nm with a Cary recording spectrophotometer. NADP and NADPH were measured by a method of alkaline extraction and enzymic cycling as described previously. The alkaline extraction led to release of dinucleotide from proteins and allowed determination of a value that represented the concentration of both protein-bound and unbound dinucleotide. Preliminary ultrafiltration allowed discrimination between bound and unbound dinucleotide. NAD and NADH concentrations were determined by use of the same extraction procedure, but with glutamate dehydrogenase and lactate dehydrogenase as cycling enzymes. The destruction of NAD in strong alkali gave the final fluorescent product for NAD determination.

A 10-mL sample of heparinized blood from a man with the Mediterranean variant of glucose-6-phosphate dehydrogenase (0-glucose 6-phosphate: NADP+1-oxidoreductase, E.C. 1.1.1.49) was filtered through cellulose for removal of leukocytes and platelets. One volume of washed, packed erythrocytes was mixed with three volumes of Krebs-Ringer/Tes buffer that contained glucose at a final concentration of 5 mmol/L. An identical preparation contained glucose oxidase at a final concentration of 9 nmol/L. At the end of the incubation, measurements were made of the proportion of the volume occupied by packed cells (hematocrit), the activity of catalase, and the concentrations of nicotinamide dinucleotides (NAD and NADP).

The erythrocytes that had been incubated with glucose oxidase were promptly centrifuged at 3,000 g for five minutes; the packed cells were mixed with 6 vol distilled water at 0°C. After ten minutes, the preparation was centrifuged for 15 minutes at 16,000 g. Four volumes of the supernatant, stroma-free hemolysate were mixed with one vol 0.75 mol/L NaCl; and 7 mL solution, containing 240 mg hemoglobin, was applied to a Sephacryl S-200 column that had been equilibrated with Krebs-Ringer/Tes buffer. Fractions of 7 mL were collected at a flow rate of 7 mL/h.

RESULTS

Relationship between rate of generation of H2O2 and rate of evolution of 14CO2. The use of labeled glucose and different amounts of glucose oxidase in the presence of normal and acatalasemic human erythrocytes led to a stoichiometric relationship between the rate of generation of H2O2 and the degree of stimulation of the HMS. Each molecule of glucose-6-phosphate (G6P) passing through the shunt results in two molecules of NADPH, which are sufficient to generate four molecules of GSH from oxidized glutathione. Since only two molecules of GSH are necessary to remove one molecule of H2O2, one molecule of glucose will remove two molecules of H2O2. Table 1 shows the rates of observed and expected 14CO2 evolution in acatalasemic and normal erythrocytes at different flows of H2O2. In acatalasemic erythrocytes ~90% of the generated H2O2 was removed by glutathione peroxidase. However, for normal erythrocytes, at each of the different rates of generation of H2O2, the observed 14CO2 evolution corresponded to ~50% of the expected values (Table 1); these values differed by >2 SD from the rate observed in acatalasemic erythrocytes.

Relationship between rate of generation of H2O2 and rate of decrease of GSH in G6PD-deficient erythrocytes. Since no stimulation of the HMS occurs in G6PD Mediterranean erythrocytes in the presence of oxidizing agents, these cells are a model for evaluation of the relationship between the...
the catalase peak shown in activity was the same in rate of decrease of erythrocytic GSH, which cannot be obtained by dividing the activity of catalase in each fraction less decrease of GSH was catalase or 6.8 nmol Fig 2 contained catalase fractions of shown in Fig 2, 76% was represented by NADPH, 16% was the results obtained with normal cells.

catalase peak dinucleotide was present in the peak. The NADPH in the regeneration after its oxidation, and the rate of generation of H202. This finding is in agreement with rates

closest to the concentration of NADPH observed in the erythrocytes (5.4 μmol/L) immediately after the cells had been incubated in the presence of glucose oxidase (Table 3).

Table 3 shows the concentrations of the four dinucleotides after incubation. The concentration of NADPH fell to less than half the value for cells incubated without glucose oxidase. Catalase activity decreased ~30%. Similar results were obtained when the experiment was performed on the cells of a second subject with G6PD deficiency whose erythrocytes were incubated in the presence of α-naphthol, an oxidizing agent, at a final concentration of 200 μmol/L.

DISCUSSION

The contribution of catalase toward the protection of the erythrocytes has been a subject of controversy for many years. Soon after the identification of glutathione peroxidase by Mills,20 some investigators suggested that this was the enzyme responsible for the activity earlier ascribed to catalase. Cohen and Hochstein4 determined the relative contributions of catalase and glutathione peroxidase and concluded that glutathione peroxidase is the major route for H2O2 breakdown under physiologic conditions, provided that GSH is available through the operation of the HMS. At higher concentrations of H2O2, the action of catalase becomes increasingly important. These conclusions were derived mainly from diffusion studies in which suspensions of intact erythrocytes were exposed to gaseous H2O2. Although the technique is intrinsically valid, the rate of entry of H2O2 into a suspension depends on many variables, such as temperature, rate of shaking, and the geometry of the incubation flask. Even with shallow suspensions, the rate of destruction of H2O2 is so rapid, relative to the rate of diffusion of H2O2, that the steady-state concentration of H2O2 can be expected to vary greatly with the distance below the meniscus. As shown by Cohen and Hochstein, ~40% to 60% of the H2O2 transferred to the main compartment is localized in the vapor droplets on the side walls.21 Therefore, for stoichiometric

rate of decrease of erythrocytic GSH, which cannot be regenerated after its oxidation, and the rate of generation of H2O2. The results in Table 2 indicate that the observed decrease of GSH was less than that expected at different rates of generation of H2O2. This finding is in agreement with the results obtained with normal cells.

Evaluation of the role of NADH in intracellular protection of catalase. Of the dinucleotides in the catalase peak shown in Fig 2, 76% was represented by NADPH, 16% was represented by NADP+ and only 8% was represented by NADH. The ratio of the dinucleotide content to catalase activity was the same in each of the four fractions representing the catalase peak shown in Fig 2. An estimate of molar concentration of human catalase in each fraction was obtained by dividing the activity of catalase in each fraction by the specific activity of highly purified human catalase (3.5 x 10^-7 mol/L/s). These calculations indicate that the catalase fractions of Fig 2 contained a total of 1.7 nmol catalase or 6.8 nmol NADP-binding sites. A total of 6.6 nmol dinucleotide was present in the peak. The NADPH in the catalase peak shown in Fig 2 represented 5.3 nmol/mL erythrocytes or 5.3 μmol/L. This concentration corresponds closely to the concentration of NADPH observed in the erythrocytes (5.4 μmol/L) immediately after the cells had been incubated in the presence of glucose oxidase (Table 3).

Table 3. Content of GSH Within Mediterranean G6PD-Deficient Erythrocytes During Exposure to Different Rates of H2O2 Production

<table>
<thead>
<tr>
<th>H2O2 Rate (nmol/h)</th>
<th>GSH Found (nmol)</th>
<th>∆ GSH Found (nmol)</th>
<th>∆ GSH Expected (nmol)</th>
<th>∆ GSH Found/∆ GSH Expected</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (Baseline)</td>
<td>656 ± 24</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>239 ± 12</td>
<td>464 ± 28</td>
<td>−192 ± 5</td>
<td>−479 ± 25</td>
<td>0.40 ± 0.03</td>
</tr>
<tr>
<td>493 ± 21</td>
<td>353 ± 37</td>
<td>−303 ± 49</td>
<td>−987 ± 42</td>
<td>0.31 ± 0.06</td>
</tr>
<tr>
<td>951 ± 37</td>
<td>272 ± 45</td>
<td>−384 ± 45</td>
<td>−1,901 ± 74</td>
<td>0.21 ± 0.03</td>
</tr>
</tbody>
</table>

The rates of 14CO2 evolution from [1-14C] and [2-14C] glucose are not shown since the differences from the baseline value were negligible even at the maximum H2O2 flow.
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of a hemolysate after chromatography in Sephadryl fractions 5-200. Catalase in disposing of H2O2. In support of the claim of the constant flow of H2O2 and is inadequate for precise evaluation of the relative roles of glutathione peroxidase and catalase in disposing of H2O2. In support of the claim of the preeminence of the NADPH/glutathione peroxidase mechanism was the observation that G6PD-deficient subjects are particularly prone to develop acute hemolytic anemia after exposure to oxidizing agents.

Other investigators reached different conclusions. Jacob et al.,22 studying acatalasemic subjects, suggested the possibility that catalase is a first, but not indispensable, line of defense against H2O2 in erythrocytes. They reported for the first time that acatalasemic erythrocytes metabolize glucose through the NADPH/glutathione peroxidase pathway.

Table 3. Effect of H2O2 on Catalase Activity and Intracellular Dinucleotide Concentration of G6PD-Deficient Erythrocytes

<table>
<thead>
<tr>
<th>H2O2 (nmol/h/ml)</th>
<th>Catalase (kU/ml Hb)</th>
<th>NADPH</th>
<th>NADH</th>
<th>NADP⁺</th>
<th>NADH⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.262</td>
<td>45.2</td>
<td>16.8</td>
<td>52.8</td>
<td>2.9</td>
</tr>
<tr>
<td>400</td>
<td>0.180</td>
<td>60.5</td>
<td>5.4</td>
<td>54.9</td>
<td>2.0</td>
</tr>
</tbody>
</table>

*The mean for normal erythrocytes from ten subjects was 2.3 ± 0.5.

Table 2 suggests that glutathione peroxidase disposed of 20% to 40% of the generated H2O2, the proportion of 20% being obtained at the higher rate of generation of H2O2 (Table 2).

The present results on normal and G6PD-deficient erythrocytes clearly indicate an active role of catalase in the removal of H2O2. Expected values for glutathione peroxidase involvement are derived from the rate of generation of H2O2. The possibility of incomplete diffusion of H2O2 through the erythrocyte membrane or of an underestimation of 14CO2 evolution is unlikely, owing to the results observed in acatalasemic erythrocytes.

The validity of conclusions from this study rests on the accuracy of estimates of rates of H2O2 production and HMS activity. Phenol, aminoantipyrine, and horseradish peroxidase were present at low concentrations in the continuous clearly demonstrated the active role of catalase in H2O2 breakdown.

The discovery of the presence and function of catalase-bound NADPH23 allows for the first time a unification of these two different mechanisms for disposing of H2O2. A reevaluation of the involvement of both mechanisms is appropriate at this time. With the technique of H2O2 generation used in the present study, the appropriate use of glucose and glucose oxidase gives a constant rate of generation of H2O2 throughout the incubation period. When coupled with measurements of the generation of 14CO2 from 14C-labeled glucose, this method provides a means of establishing the involvement of the NADPH/glutathione peroxidase system in removing H2O2. The reliability of this technique is demonstrated by the almost constant ratio of 1:1 between the observed and the expected HMS activity of catalasemic erythrocytes (Table 1). The result indicates that essentially all of the generated H2O2 enters the RBC and is inactivated stoichiometrically through the NADPH/glutathione peroxidase pathway.

When normal erythrocytes are exposed to the same rates of generation of H2O2, the activity of the HMS is almost 50% of the expected values (Table 1). Since NADPH is the common hydrogen donor for both systems, glutathione peroxidase probably accounts for even less than 50% of the expected rate (Table 1). These results are in accordance with earlier observations of Jacob and Jandl,22 who reported potentiation of the stimulation of the HMS by blocking catalase function with inhibitors, such as cyanide or sodium azide. These agents, however, neither do completely inhibit catalase nor have other metabolic effects that prevent quantitative studies.

Further evidence of the active role of catalase is easily observed in Mediterranean G6PD-deficient erythrocytes. These cells carry a baseline HMS activity that is barely sufficient to avoid overt chronic hemolysis, their maximum G6PD activity being almost nil.11,24 As reported in several articles,11,23 no stimulation of the HMS of such G6PD-deficient erythrocytes occurs in the presence of oxidizing agents. Moreover, reduction of oxidized glutathione is negligible.26 An estimate of the intracellular involvement of glutathione peroxidase is made possible by exposure of these cells to H2O2 at controlled rates of generation of H2O2.

The results shown in Table 2 suggest that glutathione peroxidase disposed of 20% to 40% of the generated H2O2, the proportion of 20% being obtained at the higher rate of generation of H2O2 (Table 2).
assay of H$_2$O$_2$ production by glucose oxidase in cell-free solution, but they were omitted from cell suspensions. If any of these three reagents were inhibitors of glucose oxidase, the rate of H$_2$O$_2$ production would have been underestimated. We found, however, that estimates of the amount of H$_2$O$_2$ produced during a period of incubation of glucose and glucose oxidase were the same whether these three reagents were added at the beginning or at the end of the incubation.

An alternative method using homovanillic acid and horseradish peroxidase yielded the same results. In their classic study of metabolism in human erythrocytes, Brin and Yonemoto demonstrated that >90% of evolved CO$_2$ comes from the first carbon of glucose when the cells are incubated without substances that stimulate the HMS. They noted that CO$_2$ is evolved from the second carbon of glucose as well when cells are incubated in the presence of methylene blue, which greatly stimulates HMS activity. Oxidation of the second carbon results from recycling through the HMS. Brin and Yonemoto showed that $\leq 5\%$ of the CO$_2$ arose from other carbons of glucose, even when methylene blue was present. Thus, the use of [1 - $^{14}$C] glucose and [2 - $^{14}$C] glucose in the present study provided an accurate measurement of HMS activity.

Conclusions from comparisons of the rates of H$_2$O$_2$ production and HMS activity would have also been in error if more than a small fraction of the generated H$_2$O$_2$ was consumed in oxidizing cellular components, such as hemoglobin. That the A/B ratio was close to 1 in the acatalasemic subject (Table 1) indicates that very little of the generated H$_2$O$_2$ was consumed in this manner in those cells. As expected from their excellent defenses against peroxidative injury, normal erythrocytes exhibited no increase in methemoglobin or in malonyldialdehyde when samples of normal erythrocytes were incubated at the highest concentration of glucose oxidase under the conditions shown in Table 1. In contrast to normal and acatalasemic cells, G6PD-deficient cells would be expected to undergo oxidation of cellular components. It is noteworthy in this connection that the ratios of G6PD-deficient cells shown in Table 2 are less than the ratios shown for normal cells in Table 1. Consideration was also given to whether the glucose in the cell suspensions was depleted or was lowered in concentration enough to slow the rate of H$_2$O$_2$ production. The Embden-Meyerhof rate of human erythrocytes in Krebs-Ringer/Tes buffer, pH 7.4, is 0.8 to 1.0 $\mu$mol/h/mL. Coupled with the HMS rates shown in Table 1, the rate of glucose consumption by the 0.5 mL erythrocytes in the 2.3-mL incubation mixture can be calculated to have been 0.5 to 0.95 $\mu$mol/h. The addition of the rate of glucose consumption by glucose oxidase (Table 1) brings the total to 0.5 to 1.85 $\mu$mol/h. This would have resulted in consumption of only 5% to 20% of the glucose during the one-hour incubation and would have caused the average rate of H$_2$O$_2$ production to be reduced by less than 2% to 10%. Actual postincubation determinations of glucose in suspensions of normal erythrocytes under the conditions of Table 1 confirmed the assumption that <20% of the glucose was consumed.

In a previous study, we demonstrated that NADH may also prevent the inactivation (through formation of compound II) of catalase during exposure of purified catalase to H$_2$O$_2$. Therefore erythrocytic NADH might have been a suitable hydrogen donor for keeping catalase functionally active even without an adequate NADPH-generating system (as in the case of G6PD-deficient erythrocytes). Figure 2 indicates, however, that very little bound NADP* was displaced by NADH when intact erythrocytes were subjected to H$_2$O$_2$ generation at a rate of 400 nmol/mL/h. Earlier studies demonstrated the stability of catalase-bound nicotinamide dinucleotides during chromatography and storage. The concentration of NADH in erythrocytes was too low to affect the displacement of NADP* from the catalase molecule, and inactivation of catalase occurs (Table 3). Administration of primarque to G6PD-deficient subjects produces decrease of ~35% in catalase activity within several days.

In conclusion, the present investigation indicates that the two routes for H$_2$O$_2$ breakdown, namely catalase and glutathione peroxidase, are equally involved in the removal of H$_2$O$_2$ in human erythrocytes. Failure of only one of the two mechanisms for disposing of H$_2$O$_2$ may not be deleterious. Since both mechanisms are dependent on the generation of the NADPH (Fig 1), however, failure of the NADPH-generating system (as with G6PD deficiency) makes the erythrocytes particularly susceptible to oxidative hemolysis.

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Catalase and glutathione peroxidase are equally active in detoxification of hydrogen peroxide in human erythrocytes

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