Active Involvement of Catalase During Hemolytic Crises of Favism

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The endemic occurrence of favism in certain Mediterranean regions provided an investigative opportunity for testing in vivo the validity of claims as to the role of catalase in protecting human erythrocytes against peroxidative injury. Reduced activity of catalase was found in the erythrocytes of six boys who were deficient in erythrocytic glucose-6-phosphate dehydrogenase (G6PD) and who were studied while suffering hemolysis after ingesting fava beans. Activity of catalase was further reduced when their red blood cells were incubated with aminotriazole. In contrast, minimal reduction of catalase activity was found, both with and without incubation with aminotriazole, in erythrocytes of a G6PD-deficient boy who had ingested fava beans 7 days earlier and in erythrocytes of seven G6PD-deficient men with a past history of favism. These results confirmed earlier studies in vitro indicating that catalase is a major disposer of hydrogen peroxide in human erythrocytes and, like the glutathione peroxidase/reductase pathway, is dependent on the availability of reduced nicotinamide adenine dinucleotide phosphate (NADPH). The effect of divicine on purified catalase and on the catalase of intact G6PD-deficient erythrocytes was similar to the previously demonstrated effect on catalase of a known system for generating hydrogen peroxide. This effect of divicine strengthens earlier arguments that divicine is the toxic peroxidative component of fava beans.

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PEOPLE WHO HAVE a deficiency of glucose-6-phosphate dehydrogenase (G6PD) are prone to acute hemolytic anemia (AHA) on exposure to certain substances, such as fava beans, primaquine, and nitrofurantoin. The traditional explanation for the AHA triggered by these substances is that G6PD-deficient cells are unable to generate enough nicotinamide adenine dinucleotide phosphate (NADPH) to maintain high levels of reduced glutathione (GSH). GSH is used by the glutathione peroxidase (GSH-Px)/reductase system to detoxify H2O2 and organic peroxides within the cell provided that sufficient GSH is made available by the hexose monophosphate shunt (HMS), of which G6PD is the first and rate-limiting enzyme. In recent years, however, catalase has gained new consideration in cellular defense, based on the discovery that each tetrameric molecule of mammalian catalase has four molecules of tightly bound NADPH that prevents and reverses the accumulation of Compound II, the inactive form of catalase. These and other findings showed that both mechanisms are dependent on NADPH generation and that a failure in the generation of NADPH, as in G6PD deficiency, impairs both systems for H2O2 detoxification. These findings brought a unity to the concept of two different mechanisms for disposing of H2O2. Evidence for the role of catalase in protecting human erythrocytes from peroxidative injury, however, has come largely, if not entirely, from experiments in vitro. In the early years of research on G6PD deficiency, much was learned from studying volunteers who took primaquine. With the present knowledge of the predictable and harmful consequences of primaquine exposure, such studies are no longer ethically justifiable.

Favism in many ways resembles drug-induced hemolysis, but differs from it in that only some G6PD-deficient subjects appear to be sensitive to fava beans. It is confined mainly to Mediterranean countries with a prevalence of the GdMed gene, although sporadic cases carrying other G6PD mutations (GdA-; GdAures) have been recently reported. Efforts to educate people about the danger of eating fava beans have reduced the incidence of favism in these Mediterranean areas. Nevertheless, sufficient cases occur to test in vivo the validity of claims as to the role of catalase in protecting human erythrocytes against peroxidative injury. In the present report, we offer such evidence. Moreover, we have observed an active participation of catalase under conditions of oxidative stress in vitro caused by the addition of divicine, a compound previously suspected of being one of the components of fava beans leading to AHA in G6PD-deficient people.

MATERIALS AND METHODS

In vivo studies. Favism was diagnosed as an acute hemolytic episode occurring after ingestion of fava beans, with anemia, hemoglobinuria, and jaundice. Blood was drawn before transfusion from seven boys having AHA of various degrees after fava beans ingestion (see Table 1) and from seven healthy G6PD-deficient (GdMed) adult men with a past history of favism. Leukocytes and platelets were removed by the method of Beutler et al. Plasma was removed after each sample was centrifuged, and the erythrocytes were divided into two portions: one was washed twice by suspension in 0.15 mol/L NaCl, and the other in 5 volumes of 0.15 mol/L NaCl containing 20 mmol/L 3-amino-1,2,4-triazole (AT), a catalase inhibitor, which in the presence of H2O2, forms an irreversible complex with the enzyme. The first portion was promptly assayed for catalase and for activity of G6PD, 6-phosphogluconate dehydrogenase, and GSH-Px by the methods of Beutler.

Catalase activity was measured by the method of Aebi and expressed as k/g Hb. The other portion of packed erythrocytes was mixed with 3 volumes of Krebs-Ringer solution/20 mmol/L Tet (N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid) buffer (pH 7.4)/5 mmol/L glucose (KRTG) as previously described, and AT was added to a final concentration of 20 mmol/L. After a 60-minute incubation in vitro indicating that catalase is a major disposer of hydrogen peroxide in human erythrocytes and, like the glutathione peroxidase/reductase pathway, is dependent on the availability of reduced nicotinamide adenine dinucleotide phosphate (NADPH). The effect of divicine on purified catalase and on the catalase of intact G6PD-deficient erythrocytes was similar to the previously demonstrated effect on catalase of a known system for generating hydrogen peroxide. This effect of divicine strengthens earlier arguments that divicine is the toxic peroxidative component of fava beans.
a metabolic shaker at 37°C at 120 oscillations per minute, the erythrocytes were collected, washed in 5 volumes of 0.15 mol/L NaCl, and assayed as described above. AT and all other reagents were from Sigma (St Louis, MO). All incubations and determinations were in duplicate.

In vitro studies with intact erythrocytes and divicine. The effect of one of the metabolites present in fava beans, 2,5-diamino-4,5-dihydroxypyrimidine (divicine), was evaluated in vitro on intact G6PD-deficient red blood cells (GdMsd) from four healthy male donors. DNA analysis of the Gd gene of some of these subjects indicated that they were carriers of the Gd<sup>red</sup> mutation. Divicine was obtained from vicine (Serva, Germany), a glycoside which, through the action of a β-glicosidase (Boheringer, Mannheim, Germany) in Tris-HCl, 10 mmol/L, pH 6.0, splits the glycosidic bond of vicine with formation of one molecule of glucose and one of divicine. After completion of this reaction, the sample was filtered with a CF-25 ultrafiltration cone (Amicon, Beverly, MA); the amount of divicine in the ultrafiltrate was measured by the amount of glucose generated from an enzymatic reaction using NADP, adenosine triphosphate (ATP), and the enzymes hexokinase and G6PD. Erythrocytes were incubated in a metabolic shaker at 37°C in KRTG buffer, in the presence of divicine (200 μmol/L), with or without AT (20 mmol/L). Erythrocytes without divicine, but with AT, served as controls. Catalase activity and GSH concentration were measured every 30 minutes for 90 minutes.

In vitro studies with purified catalase and divicine. Human purified catalase was prepared as previously reported. The interaction between divicine and catalase was followed in a spectrophotometer at 435 nm and at 37°C for 120 minutes. Each cuvette contained, in the following final concentration: KRT<sub>e</sub> buffer, pH 7.4, 2 nmol/mL of human purified catalase, β-glicosidase (2U/mL), vicine (200 mmol/mL), and a NADPH regenerating system formed by the addition of glucose-6-phosphate (600 mmol/mL), NADP<sup>+</sup> (2 nmol/mL) and G6PD (5 μg/mL). The G6PD was added at different times (see Fig 3, curves a and b).

## RESULTS

Admission to the hospital and blood sampling of the seven boys occurred within 24 to 48 hours after ingestion of the fava beans, except for patient no. 7, who had ingested the beans 7 days earlier (Table 1). All subjects showed signs of hemolysis, such as reduced hemoglobin (Hb) level, high reticulocyte count, and increased level of total bilirubin (Table 1). Erythrocytic enzyme activities and GSH were measured during AHA and before transfusion. Increased G6PD and lower concentration of GSH were consistently observed (Table 2), as reported earlier, whereas mean catalase activity was significantly decreased (P < .0002) compared with the values of G6PD-deficient subjects without AHA (Fig 1 and Table 2). In two cases, catalase activity and GSH concentration, when measured in older erythrocytes fractionated by gravity, were barely detectable. Values of GSH-Px were slightly increased. When erythrocytes collected from patients during acute hemolytic crisis were preincubated with AT for 60 minutes, catalase activity was significantly lower (Fig 1 and Table 2, P < .0003). Patient no. 7, who had ingested fava beans 7 days earlier, had an unchanged catalase value (Fig 1).

Incubation of G6PD-deficient erythrocytes in the presence of divicine, obtained enzymatically from vicine, caused a decrease of catalase activity, which was more pronounced in the presence of AT (Fig 2A). A similar behavior was observed for intracellular GSH (Fig 2B). Higher concentrations of divicine caused complete inactivation of catalase and oxidation of GSH in a shorter period of time.

The effect of divicine on purified human catalase was also evaluated. Catalase was exposed to divicine, progressively generated in the native form by the action of β-glicosidase on vicine. As observed in Fig 3 (curve a), generation of divicine caused the formation of Compound II, the inactive form of catalase, whereas the addition of a NADPH generating system (G6PD) reversed the reaction (Fig 2, second part of curve a). The simultaneous addition of divicine and the NADPH generating system protected catalase from inactivation (Fig 3 curve b).

## DISCUSSION

The catalase reaction consists of the conversion of two molecules of H<sub>2</sub>O<sub>2</sub> to one molecule of oxygen and two molecules of water. Catalase becomes an enzyme-substrate complex called Compound I on reacting with the first molecule of H<sub>2</sub>O<sub>2</sub>. Reaction with the second molecule of H<sub>2</sub>O<sub>2</sub> brings catalase back to its initial state. In the absence of bound NADPH, however, Compound I is capable of becoming Compound II, an inactive form of catalase. Compound II reverts to active catalase spontaneously, but a steady-state ratio of Compound I and Compound II exists when catalase is constantly exposed to H<sub>2</sub>O<sub>2</sub> in the absence of NADPH. Moreover, existence of catalase in the Compound II state is accompanied by an increased rate of irreversible inactivation of the catalase. As a consequence of the ability of catalase to regain activity spontaneously, the extent of catalase involvement during oxidative stress in vivo must have been even greater than is reflected in the reduced activity of the erythrocytic catalase of subjects with AHA from favism (Ta-
Table 2. Mean Values (±SD) of Catalase and G6PD Activities and GSH Level of Erythrocytes From Six G6PD-Deficient Subjects With Acute Hemolysis After Fava Beans Ingestion Compared With Seven G6PD-Deficient Controls Without Hemolysis

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<th>Controls</th>
<th>Subjects With AHA</th>
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<td></td>
<td>Plain</td>
<td>After Incubation With AT*</td>
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<tr>
<td>Catalase (k/g Hb)</td>
<td>328 ± 35</td>
<td>312 ± 28</td>
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<tr>
<td>G6PD (U/g Hb)</td>
<td>0.04 ± 0.01</td>
<td>0.05 ± 0.02</td>
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<tr>
<td>GSH (μmol/g Hb)</td>
<td>3.96 ± 0.61</td>
<td>3.79 ± 0.57</td>
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* Erythrocytes were incubated with 20 mmol/L AT for 60 minutes at 37°C in KRTG, pH 7.4, before determination of enzyme activities and GSH concentrations.

The most severely affected erythrocytes had undergone hemolysis during the 1 to 2 days after ingestion of fava beans. There was a high percentage of reticulocytes (Table 1), which have higher G6PD levels (Table 2) and generate more NADPH that should prevent catalase inactivation (Fig 3, curve b). Although it was done rapidly, the erythrocytes had been washed twice with 5 volumes of saline before catalase activity was measured. In this study, erythrocytes, which had been subjected to in vivo oxidative stress, were also exposed for a period of time to AT before preparation of the hemolysates. AT is an irreversible and specific inhibitor of catalase, once the enzyme reacts with the first molecule of H2O2 with formation of Compound I. The inhibition was observed in six of the seven patients studied (Fig 1). In the one patient who had ingested fava beans 7 days earlier, erythrocytes had normal catalase activity and were refractory to AT inhibition. Even without exposure to AT, erythrocytes from subjects with AHA had a 32% reduction of catalase activity and 25% decrease of GSH, compared with G6PD-deficient subjects without AHA, and these values reached 55% and 40%, respectively, in the presence of AT (Table 2).

Two agents, among those present in fava beans, have been suspected of being responsible for the hemolytic crises in G6PD-deficient subjects. These are two glycosidic compounds, vicine and convicine, which upon splitting of the P-glycosidic bond between glucose and the hydroxyl group at the C5 level, generate the redox aglycones divicine and isouramil. Both pyrimidine derivatives, although never identified in vivo for technical difficulties, cause oxidative stress in vitro, as described by several investigators. Toxicity of divicine and isouramil is very similar: reduced divicine is believed to be oxidized to the semiquinoid free radical form by the one-electron reduction of dioxygen and subse-

Fig 1. Distribution of catalase activity of erythrocytes of G6PD-deficient controls without (●) and with (○) incubation for 60 minutes at 37°C in the presence of 20 μmol/mL AT and of erythrocytes from subjects with AHA after ingestion of fava beans, without (ⅴ) and with incubation with AT (▼). Asterisks denotes a patient (no. 7, Table 1), who ingested fava beans 7 days before hospitalization and blood sampling.

Fig 2. Mean relative activities (± standard deviation [SD]) of catalase (A) and GSH concentration (B) in G6PD-deficient red blood cells from four different male subjects without AHA, incubated in various conditions: (●) plain, (○) in the presence of divicine 200 nmol/mL, and with (▼) divicine plus aminotriazole (20 μmol/mL).
CATALASE AND FAVISM

GSH accompanied by a decrease of catalase activity during primaquine administration to G6PD-deficient subjects. Other investigators during the same period reported, through in vitro studies, an increased HMS activity of actalasemic red blood cells (Swiss type) in resting condition and during oxidative stress. Those findings did not receive large credit until Eaton et al27 reconsidered those data after showing that catalase was protected by the addition of NADPH to crude hemolysate challenged with some oxidizing agents. Through in vitro studies with normal, G6PD-deficient and actalasemic erythrocytes, we proposed that catalase and GSH-Px are equally active in the erythrocytes.15,28 More recently, by the use of a cell-free system, we obtained evidence for an even more preeminent involvement of catalase.29 The main function of GSH-Px, at least in human erythrocytes, probably involves the reduction of organic hydroperoxides rather than H2O2, and this demand can be easily met by this enzyme because of its broad specificity with respect to hydroperoxide substrates.30

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