Active Involvement of Catalase During Hemolytic Crises of Favism

By Gian Franco Gaetani, Michela Rolfo, Sara Arena, Rosa Mangerini, Gian Franco Meloni, and Anna Maria Ferraris

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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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, 2,6-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 GdMd mutation. Divicine was obtained from vicine (Serva, Germany), a glycoside which, through the action of a α-glycosidase (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.16

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.17 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 15 minutes for 90 minutes.

In vitro studies with purified catalase and divicine. Human purified catalase was prepared as previously reported.7 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: KRTes buffer, pH 7.4, 2 mmol/mL of human purified catalase, β-glycosidase (2U/mL), vicine (200 mmol/mL), and a NADPH regenerating system formed by the addition of glucose-6-phosphate (600 mmol/mL), NADPH* (2 mmol/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,17 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 1, 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-glycosidase on vicine. As observed in Fig 3 (curve a), generation of divicine caused complete inactivation 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 H₂O₂ 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 H₂O₂. Reaction with the second molecule of H₂O₂ 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 H₂O₂ in the absence of NADPH.18,19 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 Subjects With AHA</th>
<th>Controls 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 β-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. Toxin 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 (C) and with (o) 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: (C) plain, (o) in the presence of divicine 200 nmol/mL, and with (V) divicine plus aminotriazole (20 μmol/mL).
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Fig 3. Formation of Compound II (curve a) during exposure of human purified catalase to divicine generated by 2 U of β-glycosidase from vicine (200 nmol/mL) and (curve b) in the presence of a NADPH generating system formed by the addition of glucose-6-phosphate (600 nmol/mL), NADP+ (2 nmol/mL), and G6PD (5 μg/mL). Addition of G6PD after 80 minutes to reaction (curve a) promptly reversed the formation of Compound II.

quent generation of H2O2. Divicine and isouramil have been shown in vitro to cause a fall of GSH and NADPH, generation of Heinz bodies, membrane cross-bonding, and Cu2+-ATPase inhibition. Their potential toxic effect on catalase, however, had never been tested. The in vitro findings with one putative toxic metabolite present in fava beans are quite similar to the in vivo observations. During exposure of G6PD-deficient erythrocytes to divicine, used at concentration considered probably realistic after ingestion of a small amount of beans, catalase decreases its catalatic activity to less than 10% after a 90-minute incubation and about 50% of GSH is in the oxidized form (Fig 2). As with in vivo studies, these effects are more evident in the presence of AT, when inactivation of catalase seems to anticipate oxidation of GSH (Fig 2A and B). If divicine is interacting with catalase, the same phenomenon should be observed in a cell-free system containing purified human catalase. As previously reported, generation of H2O2 causes a progressive inactivation of catalase that can be reversed or prevented if adequate generation of NADPH is provided.

This phenomenon has been observed in the presence of native divicine (Fig 3, curve a) when the increased formation of Compound II is promptly stopped by addition of the NADPH generating system (Fig 3, second part of curve a). On the other hand, when there is simultaneous generation of NADPH and divicine, as occurs in erythrocytes with normal G6PD levels, the formation of Compound II is negligible (Fig 3, curve b).

The present in vivo data fully agree with the observations made by early investigators of G6PD who reported a fall in 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 al 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. More recently, by the use of a cell-free system, we obtained evidence for an even more preeminent involvement of catalase. 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.

ACKNOWLEDGMENT

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