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Favism: Disordered Erythrocyte Calcium Homeostasis

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The biochemical events that take place during acute hemolysis of G6PD-deficient subjects in favism are far from being elucidated. Evidence is here reported for a constantly and heavily disordered calcium homeostasis in the erythrocytes from seven favic patients. The abnormality, ie, a significantly impaired calcium ATPase activity and a parallel marked increase of intracellular calcium levels, was characteristic of the acute hemolytic crisis although unrelated to the attendant reticulocytosis. Concomitantly, a remarkable decrease of intracellular potassium was also observed. The mean ± SD Ca^{2+}-ATPase activity in the favic patients was 20.8 ± 7.8 μmol P_i/g Hb/h compared with 37.2 ± 8.5 in the matched controls represented by 12 healthy G6PD-deficient subjects ($P < .001$). The mean ± SD intraerythrocytic calcium content was 288 ± 158 μmol/L of erythrocytes in the favic patients as compared with 22.0 ± 8.2 in the G6PD-deficient controls ($P < .001$). The intraerythrocytic potassium content was 76.6 ± 19.3 mmol/L of erythrocytes in the favic patients and 106.6 ± 8.2 in the G6PD-deficient controls ($P < .001$). In vitro incubation of normal and G6PD-deficient erythrocytes with divicine, a pyrimidine aglycone present in fava beans and strongly implicated in the pathogenesis of favism, reproduces most of these events, including drop of calcium ATPase, increased intracellular calcium, and leakage of erythrocyte potassium.

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THE BIOCHEMICAL mechanisms that mediate oxidative hemolysis in genetically glucose 6-phosphate dehydrogenase (G6PD)-deficient erythrocytes are still poorly understood. Specifically, the sequence of events taking place during the acute hemolytic crisis induced by fava beans in persons having low activity variants of this enzyme has not been defined so far, in spite of several attempts made in this area. Two pyrimidine aglycones, divicine and isouramil, have been strongly implicated as the toxic components of fava beans,[1-4] and evidence has indeed been accumulating for a number of damaging effects induced by either compound on G6PD-deficient erythrocytes through still undefined oxidizing mechanisms.[2,3,5] A powerful oxidant, phenylhydrazine, has been shown to impair a crucial function of the membrane of normal erythrocytes, ie, extrusion of calcium.[6] Prompted by this finding, we have now explored calcium homeostasis in favism and have found abnormally elevated levels of intraerythrocytic calcium and parallel decreases of calcium ATPase activity in the erythrocytes from seven patients during acute hemolytic crisis. Comparable results, ie, imbalance of the calcium pump and concomitant accumulation of intracellular calcium, have been obtained following the in vitro exposure of normal and of G6PD-deficient erythrocytes to divicine.

MATERIALS AND METHODS

Blood samples and biochemical assays. Blood samples from 28 men were drawn and investigated according to the principles of the Helsinki declaration. Of these subjects, six with normal G6PD activity had the normal wild-type enzyme (B). The other 22, of Sardinian ancestry, were all G6PD-deficient as assessed by the spectrophotometric assay described previously,[7] their levels of erythrocyte G6PD activity ranging between 0.001 and 0.012 IU/g Hb. Of these 22 subjects, seven (five children and two adults) were favic patients during acute hemolytic crisis, as indicated by severe anemia, overt hemoglobinuria, reticulocyte counts above 5%, and methemoglobin levels over 5%. Three additional patients had suffered a hemolytic episode upon ingestion of fava beans from five to eight days previously. Finally, 12 G6PD-deficient subjects were asymptomatic, as assessed by normal hematologic parameters including packed cell volume (PCV) above 0.40, mean corpuscular volume (MCV) between 83 and 95 fL, reticulocyte counts below 1.3%, serum bilirubin levels below 1 mg/dL, and lack of hemoglobinuria. These asymptomatic G6PD-deficient subjects represented controls for this study or were donors of blood samples for the in vitro experiments.

Blood samples were drawn, using heparin as anticoagulant, and were processed immediately for measurements of calcium and potassium levels, or within 24 hours, for assays of Ca^{2+}-ATPase activity. They were centrifuged, and the buffy coat was discarded after each of three washes with 3 vol of ice-cold 150 mmol/L NaCl, preliminarily filtered on a 10 × 2-cm column of Chelex-100 ion exchange resin (Bio-Rad Laboratories, Richmond, Calif), in order to remove contaminating calcium. The washing solution was prepared before each measurement and was verified to be calcium-free as follows. Aliquots of 0.5 mL of washed erythrocytes and of the washing solution were mixed with 1 mL of 10% trichloroacetic acid (TCA) containing 10,000 ppm lanthanum. Calcium and potassium were determined in the corresponding supernatants or solutions using atomic absorption spectrophotometry with a nitrous oxide/acetylene or an air/acetylene flame, respectively.[6,8]

Assays of Ca^{2+}-ATPase were carried out on hemolysates obtained upon submitting the washed erythrocytes at a final 10% hematocrit in NaCl-Histidine buffer to three cycles of freezing–thawing.[9] Assays of Ca^{2+}-ATPase were carried out according to the procedure of Hanahan and Ekholm,[9] modified in that incubations were for 30 minutes at 37 °C. In some assays of Ca^{2+}-ATPase, purified calmodulin (either from bovine brain or from human erythrocytes, purchased from Sigma Chemical Co, St Louis) was added at concentrations from 2 to 20 μg/mL of incubation mixture. Reticulocyte counts were made on dry preparations after supravital staining; at least 1,000 cells were counted.

Fractionation of erythrocytes according to density was obtained by centrifugation on discontinuous gradients of Stractan II,[10] as described.[11] Four cell layers were recovered, which were defined as fractions 1, 2, 3, and 4, respectively, in order of increasing density.

In vitro studies with divicine. Washed erythrocytes from asymptomatic G6PD-deficient individuals and from normal controls

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were incubated under sterile conditions at a final 10% hematocrit in 0.04 mol/L *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES), pH 7.4, containing NaCl up to an osmolarity of 310 mosm/L H_2O (isoosmotic TES–NaCl), in the presence of gentamycin (50 μg/mL), 5 mmol/L glucose, 0.2 mmol/L ascorbate, and 2 mmol/L divicine. The final volume of each incubation mixture was 40 mL. Divicine was obtained immediately before each experiment by hydrolyzing commercial vicine (Serva, Feinbiochemica GMBH, & Co, Heidelberg, FRG) with 1 N HCl for 20 minutes at 100 °C, followed by rapid cooling in ice water and adjustment of pH to 7.4. Control experiments lacking ascorbate and divicine were constantly run in parallel. After the erythrocyte suspensions were incubated under gentle stirring in glass flasks for three hours at 37 °C, samples were withdrawn for assays of Ca^{2+}-ATPase and of erythrocyte calcium and potassium. The erythrocytes (both treated with divicine and untreated) were then washed three times in isoosmotic TES–NaCl and reincubated at 37 °C for periods up to 60 hours in the same buffered solution containing 0.3 mmol/L $CaCl_2$, 10 mmol/L glucose and gentamycin (50 μg/mL) at a final 10% hematocrit. At various time intervals, aliquots were submitted to assay of Ca^{2+}-ATPase activities and of erythrocyte calcium and potassium.

RESULTS

Ca^{2+}-ATPase activity and the intracellular calcium and potassium contents were explored in a number of hemizygous G6PD-deficient males during an acute hemolytic crisis triggered by ingestion of fava beans. Because high reticulocyte counts were invariably present in these subjects, preliminary experiments were carried out in order to establish the influence of varying amounts of reticulocytes on the biochemical properties under study. These experiments were performed on erythrocyte fractions separated according to densities that have significantly different reticulocyte counts (Table 1). Although the data presented in Table 1 refer to values obtained from healthy G6PD-deficient subjects (see Materials and Methods section), similar conclusions were obtained using density-fractionated erythrocytes from normal individuals having G6PD B. On the whole, the intracellular calcium content does not show significant variations in the different cell layers, in agreement with earlier observations of Wiley and Shaller.[13] On the contrary, the erythrocyte potassium levels decayed progressively from the top to the bottom cell layers in the density gradient and the same behavior was observed for Ca^{2+}-ATPase activity, thus confirming previous findings by other investigators[9,14] (Table 1). Accordingly, the contribution of a high reticulocytosis should be an enhanced intracellular content of potassium and an increased Ca^{2+}-ATPase activity.

Table 1. Levels of Ca^{2+}-ATPase and Erythrocyte Calcium and Potassium Content in Density-Separated Erythrocyte Fractions From Healthy G6PD-Deficient Subjects

Density Fractions	Reticulocytes (%)	Ca^{2+}-ATPase (μmol P_i/g Hb/h)	Intracellular Calcium (μmol/L)	Intracellular Potassium (mmol/L)
Unfractionated	0.95 ± 0.21	39.6 ± 4.3	19 ± 6	109 ± 6
1	5.72 ± 0.65	44.9 ± 4.4	21 ± 3	126 ± 12
2	1.66 ± 0.04	38.7 ± 2.9	16 ± 5	106 ± 4
3	0.22 ± 0.05	31.6 ± 1.7	17 ± 4	97 ± 3
4	—	28.4 ± 2.6	22 ± 3	83 ± 6

Mean ± SD of values from three asymptomatic G6PD-deficient subjects. Blood samples were defibrinated and erythrocytes freed of contaminating leukocytes and platelets according to Beutler et al.[12] Paired measurements on unfractionated erythrocytes obtained, using heparin as anticoagulant, gave essentially superimposable results.

The data reported in Table 2 indicate that the erythrocytes from seven favic patients have a much higher intracellular calcium content and, in spite of the elevated reticulocyte counts, both a substantially decreased Ca^{2+}-ATPase activity and lower levels of intracellular potassium, as compared with the figures observed in several healthy G6PD-deficient hemizygous subjects recruited from the same families or from the same geographic area. Changes in the three parameters proved to be highly significant from a statistical standpoint. Moreover, owing to the interference afforded by reticulocytosis, the loss of Ca^{2+}-ATPase activity and the decrease of intracellular potassium in the mature erythrocytes were, in reality, still more pronounced than they appear from inspection of Table 2. Finally, the biochemical variations under study were strictly related to the acute hemolytic crisis, since they were found to revert to normal values in the erythrocytes from favic patients shortly after remission from the acute hemolytic episode, ie, within four to five days from its clinical onset (data not shown).

The correlation between actual drop of the Ca^{2+}-ATPase activities and enhanced intracellular calcium levels, as indicated by a Pearson product-moment correlation coefficient of −0.504, is much looser than predictable on grounds of the known efficiency of this membrane protein in extruding calcium ions. This might indicate some biochemical dysfunction, affecting, for instance, the affinity of Ca^{2+}-ATPase

Table 2. Mean Levels of Ca^{2+}-ATPase, Erythrocyte Calcium and Potassium Content, Reticulocyte Counts and Methemoglobin in Favic Patients and in Asymptomatic G6PD-Deficient Controls

Subjects	Ca^{2+}-ATPase (μmol/P_i/g Hb/h)	RBC Calcium (μmol/L)	RBC Potassium (mmol/L)	Reticulocytes (%)	Methemoglobin (%)
Favic (F)					
(N = 7)					
Mean	20.8	288.0	76.6	15.43	11.2
SD	7.8	158.0	19.3	6.98	4.6
Healthy (H) G6PD-deficient					
(N = 12)					
Mean	37.2	22.0	106.6	1.08	1.2
SD	8.5	8.2	8.2	0.11	0.2
F/H	$P < .001$	$P < .001$	$P < .001$	$P < .001$	$P < .001$

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Table 3. In Vitro Effects of Divicine on Ca^{2+}-ATPase Activity and Calcium and Potassium Contents of Normal and G6PD-Deficient Erythrocytes

Erythrocytes	Time (h)	Ca^{2+}-ATPase Activity (μmol P_i/g Hb/h)		Calcium Content (μmol/L RBCs)		Potassium Content (mmol/L RBCs)	
		Normal	Gd^-	Normal	Gd^-	Normal	Gd^-
Native	0	37 ± 7	39 ± 6	17 ± 4	18 ± 7	108 ± 4	107 ± 5
Untreated	3	35 ± 6	38 ± 8	17 ± 6	17 ± 10	102 ± 9	107 ± 10
Divicine-treated	3	23 ± 4	24 ± 7	13 ± 4	10 ± 6	88 ± 6	91 ± 9
Untreated	12	33 ± 5	34 ± 4	24 ± 10	22 ± 11	91 ± 14	96 ± 8
Divicine-treated	12	10 ± 7	7 ± 4	98 ± 21	95 ± 32	63 ± 16	48 ± 24

Mean ± SD of three experiments (normal subjects having G6PD B) and of five experiments (healthy G6PD-deficient subjects), respectively. Erythrocytes were submitted to the two-step incubation procedure involving exposure to 2 mmol/L divicine and 0.2 mmol/L ascorbate for three hours, followed by reincubation in the absence of divicine and ascorbate and in the presence of 0.3 mmol/L $CaCl_2$ for 12 hours. The untreated erythrocytes were incubated in the same way, yet without divicine and ascorbate in the three-hour incubation step.

toward either calcium ions or ATP rather than its V_{max}, or alternative mechanisms such as loss of endogenous activators of this enzyme protein.[15-18] Among these, calmodulin seems not to be involved, as suggested by experiments in which addition of calmodulin itself to the hemolysates failed to activate Ca^{2+}-ATPase over the levels actually observed.

In an attempt to rationalize the picture observed in favism, G6PD-deficient erythrocytes from healthy donors (see Materials and Methods section) were exposed to an oxidant stress represented by 2 mmol/L divicine and 0.2 mmol/L ascorbate. The same oxidative challenge was exerted on normal erythrocytes, having G6PD B, for comparative purposes. As shown in Table 3, this treatment produces in both G6PD-deficient and normal erythrocytes an inactivation of Ca^{2+}-ATPase that progresses also when the oxidant mixture has been removed. Because the normal cells are fully competent to reduce their oxidized glutathione,[1,2,5,19] the decrease of Ca^{2+}-ATPase activity seems not to be a consequence of GSH oxidation and might accordingly represent a primary or metabolically unrelated event. In both types of erythrocytes, the impairment of Ca^{2+}-ATPase activity following exposure to divicine is paralleled by an increase of intracellular calcium and by a decrease of erythrocyte potassium, while hemolysis is not observed in these conditions. Over longer times of incubation (up to 60 hours) such electrolyte imbalances are still more pronounced, yet they become apparent also in the untreated erythrocytes, probably as a result of energy depletion in these conditions.[20,21]

DISCUSSION

The inactivation of Ca^{2+}-ATPase in erythrocytes exposed to a specific oxidant such as divicine and its cellular consequences, notably an abnormally elevated calcium content, could play an important role in the multistep chain of events that culminate in the massive destruction of G6PD-deficient erythrocytes taking place in favism. The loss of erythrocyte potassium that occurs concomitantly to intracellular calcium accumulation ("Gardos" effect[22]) may represent an additional mechanism of electrolyte imbalance, predisposing the erythrocyte to premature hemolysis.[23]

The correspondence between the in vitro and the in vivo findings and the time course of the phenomenon during the disease leave little doubt of the fact that disordered calcium homeostasis is a typical property of the acute hemolytic crisis in favism.[24] However, it is unlikely that the remarkable abnormalities of intracellular electrolytes that are observed in favism are the primary causes of hemolysis. In fact, they take place also in the normal erythrocytes incubated in vitro with divicine (Table 3). Moreover, no hemolysis is observed in these in vitro experiments, not even in the G6PD-deficient erythrocytes. Finally, a near-normal in vitro viability and metabolic competence has been reported for normal erythrocytes loaded with Ca^{2+} by means of the ionophore A 23187.[25] Thus, in spite of the potentially harmful biochemical events these electrolyte imbalances may trigger—for instance, sudden activation of a Ca^{2+}-dependent neutral proteinase[26]—additional mechanisms and factors seem to be involved in the process of oxidative hemolysis.[27] Experiments are in progress to identify these mechanisms and to elucidate their role in the overall process leading to erythrocyte destruction.

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