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Intracellular Restraint: A New Basis for the Limitation in Response to Oxidative Stress in Human Erythrocytes Containing Low-Activity Variants of Glucose-6-Phosphate Dehydrogenase

(glucose-6-phosphate dehydrogenase deficiency/hexose monophosphate shunt/NADPH)

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ABSTRACT **Several mechanisms recently proposed for regulation of the hexose monophosphate shunt require the concentration of NADP to be low or that of NADPH to be high. The present study indicates that the first enzyme of the hexose monophosphate shunt of human erythrocytes is under severe restraint even when these conditions do not exist. In human erythrocytes containing low-activity variants of this enzyme, glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate:NADP$^+$ 1-oxidoreductase; EC 1.1.1.49), measurements of the rate of oxidation of C-1 labeled glucose show that the enzyme is operating at a rate much closer to its maximum than in normal cells. This requires that the ratio of inhibitory NADPH to NADP be much lower in the variant cells than in normal cells. A small increase in oxidative rate, induced by naphthol, then causes a disappearance in reduced glutathione in the variant cells, presumably because a significant further decrease in NADPH occurs in these cells, whereas the same oxidative stress in normal cells would not lower the NADPH level appreciably. A low NADPH/NADP ratio in unstressed cells deficient in glucose-6-phosphate dehydrogenase is confirmed by direct measurement. The maximum activity of the variant enzyme in the cell, as measured with methylene blue to keep most of the NADP in the oxidized form, is only about 1/60 of that found in hemolysates, thus accounting for the failure to compensate for a relatively small oxidative stress *in vivo* in spite of an apparent sufficiency of enzyme. The reason for the limitation on maximum intracellular activity is unknown. A similar limitation is seen with normal cells incubated with methylene blue, where the maximum intracellular rate is also only about 1/60 of that found in hemolysates.**

Deficiencies of erythrocyte glucose-6-phosphate dehydrogenase (G6PD; EC 1.1.1.49; D-glucose-6-phosphate: NADP$^+$ 1-oxidoreductase) are among the more prevalent of hereditary enzymic defects in human beings. Subjects with these sex-linked deficiencies are susceptible to acute hemolytic anemia after exposure to primaquine, naphthalene, and certain other drugs or toxic substances. Numerous studies have led to the conclusion that the human erythrocyte must maintain a minimal level of reduced glutathione (GSH) if it is to protect itself against endogenous or induced oxidative stress (1–6). Erythrocyte GSH is regenerated from oxidized glutathione by the enzyme glutathione reductase, which requires NADPH (Fig. 1). The G6PD-deficient erythrocyte seems to be unable to provide NADPH at a rate sufficient to maintain the glutathione in a reduced form during exposure to drug-intermediates and toxic substances that place an oxidative drain on GSH.

If such is the mechanism of drug-induced hemolysis in G6PD-deficient cells, then stimulation of the hexose monophosphate shunt (HMS) should be observed in normal erythrocytes of a person who has been taking one of these drugs. In 1971, Welt *et al.* reported the results of such studies (7). When incubated with their own serum, erythrocytes of normal men exhibited higher levels of HMS activity after 3 days of primaquine ingestion than before such ingestion. The amount of stimulation of the HMS (10–20%), however, was small when compared with the observation that maximal activity of G6PD in hemolysates of G6PD-deficient men is 100 to 400 times greater than the rate at which it functions in the HMS of the resting cell.

In the present study, the HMS was measured in both normal and variant cells under conditions that simulate drug-induced, oxidative stress, and also under maximal oxidative stress induced by methylene blue. The choice of α-naphthol as the substance providing drug-induced oxidative stress stems from the knowledge that the naphthols are major

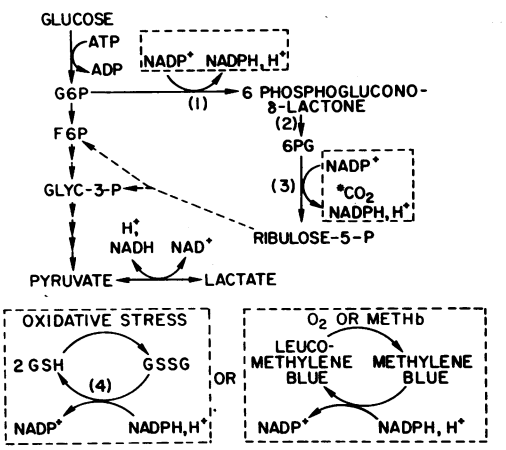

FIG. 1. Metabolic features of the hexose monophosphate shunt. (*1*) Glucose-6-phosphate dehydrogenase (G6PD); (*2*) 6-phosphogluconо-δ-lactonase or spontaneous hydrolysis; (*3*) 6-phosphogluconate dehydrogenase; (*4*) glutathione reductase. * from C-1 of glucose. *GSSG*, oxidized glutathione.

Abbreviations: G6PD, glucose-6-phosphate dehydrogenase; HMS, hexose monophosphate shunt; EMP, Embden-Meyerhof pathway; GSH, reduced glutathione.

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intermediates in the metabolism of naphthalene, the agent responsible for hemolysis in G6PD-deficient children who ingest mothballs. GSH levels fall in G6PD-deficient erythrocytes exposed to naphthols *in vitro* (4), and stimulation of the HMS of human erythrocytes occurs during incubation with large amounts of naphthol (5).

The results indicate that the cells containing low-activity variants of G6PD fail to overcome a small oxidative stress because they are already operating close to the maximum rate obtainable when all NADP is in the oxidized form. This maximum rate is only 1/60 of that expected from hemolysate measurements, thus accounting for the earlier inconsistency.

MATERIALS AND METHODS

The assay for G6PD was by spectrophotometric determination of NADPH produced by the enzyme in the presence of G6P and NADP (8). Identification of the electrophoretic variants by starch gel electrophoresis in Tris–EDTA–borate was performed as described in a W.H.O. technical bulletin (8). All blood samples (25–35 ml) were from men. G6PDA− is the variant present in most G6PD-deficient black males. G6PD Mediterranean is the most common type of G6PD deficiency in males of Greek, Sardinian, or Sephardic Jewish ancestry. Studies on all subjects were performed with defibrinated blood within 1 hr of the time of venipuncture. The blood was centrifuged at 1000 × *g* for 10 min. Serum and buffy coat were discarded. The erythrocytes were suspended in 5 volumes of 0.15 M NaCl and centrifuged again. Supernatant fluid was removed, and the erythrocytes were mixed with an equal volume of buffer, consisting of Krebs–Ringer buffer (9) that also contained glucose, at a final concentration of 5 mM, and *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) (sodium) at a pH of 7.4 and a final concentration of 5 mM (KRTG solution). The hematocrit (usually 45–50%) was determined. Incubation mixtures consisted of 1.0 ml of this erythrocyte suspension, 1.0 ml of KRTG solution (with or without dissolved α-naphthol or methylene blue), and 0.3 ml (0.125 μCi) of [*1*-^{14}C]glucose (3 μCi/μmole) in 80 mM NaCl–0.1 M TES, pH 7.4. The α-naphthol was soluble at the final concentration used (20–80 μM). All incubations with [*1*-^{14}C]glucose were performed in duplicate. For assays of reduced glutathione (GSH), [*1*-^{14}C]glucose was omitted. The incubation flask consisted of a scintillation vial fitted with a rubber cap and disposable center well, containing 0.2 ml of 2 M NaOH. The vials, covered with aluminum foil, were incubated for 6 hr at 37° in a Dubnoff metabolic shaker at 90 cycles/min. When normal and G6PD A samples were incubated with methylene blue, the incubation time was limited to 90 min so that no more than half of the 7.5 μmoles of added glucose would be consumed. At the end of the incubation, 0.7 ml of 3.7 M perchloric acid was injected by needle through the rubber cap into the incubation mixture containing [*1*-^{14}C]glucose. The vials were incubated again in the metabolic shaker for 30 min. The disposable center wells were dropped into scintillation vials containing 10 ml of Aquasol (New England Nuclear Corp.) and 0.8 ml of water, for determination of ^{14}C radioactivity.

The incubation mixture, containing perchloric acid, was neutralized with KOH, and a portion of the supernatant fluid was applied to miniature anion-exchange columns for the determination of ^{14}C-labeled anions by an adaptation (7) of the method of Rose and O'Connell (10). It provided an estimate of activity of the Embden–Meyerhof–Parnas pathway (EMP), based on the fact that 6-phosphogluconate and intermediates of the EMP are anions at neutral pH.

Glucose concentrations were measured by an enzymatic method using NADP, ATP, and the enzymes G6PD and hexokinase (11). GSH was determined as described by Beutler *et al.* (12).

TABLE 1. *Effect of α-naphthol on the mean HMS activity and final reduced glutathione content of human erythrocytes**

Subjects	α-Naphthol concentration (μM) 0	20	40	80
	HMS activity (μmoles/hr per ml of erythrocytes)			
With normal activity:				
Normal subjects (5)	0.091	0.118	0.163	0.234
G6PD A (3)	0.100	0.134	0.163	0.228
With G6PD deficiency:				
G6PD A− (3)	0.065	0.070	0.081	0.123
Mediterranean (3)	0.044	0.048	0.050	0.057
	GSH (mg/100 ml of erythrocytes)			
With normal activity:				
Normal subjects (5)	68	66	64	63
G6PD A (3)	60	59	60	56
With G6PD deficiency:				
G6PD A− (3)	50	38	31	25
Mediterranean (3)	38	28	13	9

Numbers in parentheses refer to the number of subjects of each type.

* Incubation was at 37° for 6 hr.

RESULTS

In Table 1 may be seen the effects of α-naphthol on the activity of the HMS and on the concentration of GSH in the erythrocytes of both normal and G6PD-deficient men. Erythrocytes of G6PD-deficient men had less GSH after 6 hr of incubation at 37° in the presence of α-naphthol than after 6 hr in the absence of α-naphthol. No major decline was observed in the concentration of GSH in erythrocytes with either normal G6PD or G6PD A, a prevalent electrophoretic variant in black males with nearly normal levels of activity of the enzyme. GSH levels of G6PD-deficient cells fell at concentrations of α-naphthol that gave as little as 30% stimulation of the HMS of normal cells. Among the eight normal and G6PD A subjects, the highest regression coefficient for HMS activity against α-naphthol concentration was only 2.1/hr. The highest value among the six G6PD-deficient subjects was 1.1/hr. The regression coefficient for GSH (against α-naphthol concentration) of each of the six deficient samples fell outside the range (mean ± 2 SD) of the five normal subjects.

As indicated in Table 2, methylene blue caused many-fold stimulation of HMS in normal erythrocytes and some stimulation of the HMS in G6PD-deficient erythrocytes. At a methylene blue concentration of 100 μM, the activity of the HMS in intact, normal and G6PD A cells was about 1/60 the activity of G6PD in hemolysates from the same persons. A similar ratio was observed for the two types of G6PD-deficient erythrocytes (Table 2). Less ^{14}C label appeared in the EMP intermediates when normal erythrocytes were incubated with

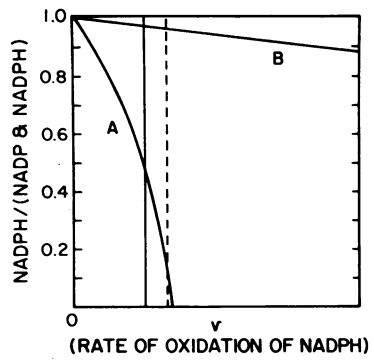

FIG. 2. Ratio of NADPH/(NADP + NADPH) predicted from Eq. 2 (*text*) for a steady-state reaction of G6PD under various rates, v, of oxidation of NADPH. Total nucleotide (NADP + NADPH) concentration, 50 μM. K_m for NADP, 3.8 μM. K_i for NADPH, 9.5 μM (15). *Curve A* is for a reaction in which the maximal rate of the enzyme (at infinite NADP concentration) is 1.5 times the baseline rate, represented by the *solid vertical line; curve B*, 20 times the baseline. A 30% increase in rate of oxidation of NADPH (from *solid* to *dashed vertical line*) results in a greater drop, and lower value, of NADPH/(NADP + NADPH) with *A* than with *B*.

methylene blue than when they were incubated without methylene blue. This has been observed by Rose and O'Connell (10) when the HMS is greatly stimulated by methylene blue. It results, at least in part, from the re-entry into the EMP of intermediates that have lost their ^{14}C in the oxidative portion of the HMS. The EMP value for the methylene blue-stimulated sample of G6PD A cells (Table 2) may not be significantly lower than that of the other, normal erythrocytes (7, 10).

DISCUSSION

This study was performed with concentrations of α-naphthol causing only slight stimulation of the HMS and corresponding to probable levels *in vivo* during naphthalene intoxication. Under more drastic conditions, when erythrocytes were depleted of GSH by exposure to azoester, Kosower *et al.* (13) and Rieber and Jaffé (14) demonstrated that intact, G6PD-deficient erythrocytes could not regenerate GSH effectively, whereas the corresponding hemolysates could do so. HMS activities were not measured. The results of Table 1, and of Kosower *et al.* (13) and Rieber and Jaffé (14), could be explained if the G6PD of resting, G6PD-deficient erythrocytes were operating close to the maximal intracellular velocity. As illustrated in Fig. 2, slight increases in demand for NADPH, of the degree shown in Table 1, should not cause serious declines in concentrations of NADPH (and therefore of GSH) if the maximal intracellular velocity is many times the resting rate. The curves of Fig. 2 were constructed from the equation for competitive inhibition:

$$v = V_{max}\, S/[S + K_m(1 + I/K_i)], \qquad [1]$$

in which S and K_m denote the concentration and Michaelis constant, respectively, for NADP, and I and K_i denote the concentrations and competitive inhibitor constant, respectively, for NADPH. G6PD catalyzes a reaction for which one of the products, NADPH, is an inhibitor and also becomes the substrate (NADP) upon being oxidized. Eq. 1 is customarily used to predict the enzymic rate, v, when the concentration S and I are defined. It can also be used to predict I, in a steady-state reaction in which NADPH is being oxidized at a defined rate v. For the latter purpose, Eq. 1 can be rearranged to:

$$\frac{I}{I+S} = \frac{V_{max} - v - K_m v/(I+S)}{V_{max} - v + K_m v/K_i} = \frac{\text{NADPH}}{\text{NADP} + \text{NADPH}}, \qquad [2]$$

where $I + S$ is a constant (50 μM in Fig. 2). Values for K_m and K_i used in Fig. 2 are those determined by Yoshida (15) for G6PD Mediterranean, but a similar relationship between the curves and resting rate occurs over a wide range of values for K_m and K_i.

Activities of G6PD in hemolysates of G6PD Mediterranean and A− subjects exceed the resting HMS rate by over 100-fold (ratio E/C, Table 2). If the maximal intracellular activity is close to the resting HMS, then a very serious reduction in intracellular activity must be presumed to exist. Even when allowance is given for known K_m and K_i values and for intracellular concentrations of substrate and possible inhibitors (15), G6PD A− and Mediterranean should be able to function at rates many times those of the resting HMS rate of erythrocytes.

The results of Table 2 also indicate that the maximal intracellular activity of G6PD is greatly reduced. In the erythro-

TABLE 2. *Comparison of G6PD activity of hemolysates with HMS and EMP activity of erythrocytes stimulated by methylene blue*

	Intact erythrocytes					
	EMP*		HMS*		Hemolysate G6PD*	
Methylene blue (μM)	(A) 0	(B) 100	(C) 0	(D) 100	(E) 0	(D/E)
Subjects						
With normal activity:						
Normal subject (1)	0.839	0.759	0.072	2.919	165	1/57
G6PD A (1)	0.995	0.393	0.081	2.960	166	1/56
With G6PD deficiency:						
G6PD A− (2)	1.212	1.101	0.066	0.529	28.4	1/54
Mediterranean (3)	0.880	0.904	0.044	0.075	5.3	1/71

Numerals in parentheses refer to the number of subjects of each type.
HMS, hexose monophosphate shunt ($^{14}CO_2$ from [1-^{14}C]glucose).
EMP, Embden–Meyerhof–Parnas pathway (^{14}C-labeled anions from [1-^{14}C]glucose).
* Mean activity (μmoles/hr per ml of erythrocytes at 37°).

cyte, methylene blue leads to rapid oxidation of NADPH by oxygen (Fig. 1). Davidson and Tanaka have shown that stimulation of the HMS by methylene blue is maximal at a methylene blue concentration of about 100 μM (16). The results of methylene blue stimulation on normal and G6PD-deficient erythrocytes indicate that, in each type of cell, the HMS activity is only 1/54 to 1/71 of the maximal activity of G6PD in hemolysates (ratio D/E, Table 2). Of significance is the observation that these ratios were similar in each of the types of cells and over a 30-fold range in G6PD activity. This finding is taken as evidence that G6PD is a rate-limiting step of the HMS of intact erythrocytes in the presence of methylene blue. True HMS activity of methylene blue-stimulated erythrocytes exceeds, by about one-half, the activity estimated from [1-^{14}C]glucose as a consequence of recycling of glucose through the HMS (5, 16, 17). True HMS activity exceeds the estimate even less when α-naphthol is present (5). The magnitude of these errors is too small to explain the large discrepancy noted here between maximal G6PD activity of hemolysates and that of intact erythrocytes.

As indicated in Fig. 2, a considerable fraction of the nucleotide should be in the oxidized form, if the G6PD of resting erythrocytes of G6PD-deficient subjects is operating close to the maximal rate. In studies using the technique of Lowry and Passonneau (18) and being reported in greater detail elsewhere, we have found only 32–47% of the NADP was in the reduced form in freshly drawn blood of four G6PD A− men. Only 16–29% was in the reduced form in the freshly drawn blood of six G6PD Mediterranean men. In contrast, over 95% of the NADP was in the reduced form in erythrocytes from 15 normal men, as has been observed by others (19). Such ratios of NADPH to total nucleotide would not be expected from known intracellular concentrations (1, 15) of substrate and inhibitors, K_m and K_i values, and hemolysate activities, unless the intracellular activities of the G6PD-deficient cells were reduced by an additional factor of about 50-fold. These observations also allow exclusion of the possibility that the impaired function of intracellular G6PD results from inhibition by α-naphthol of methylene blue. Neither substance was present during measurements of the NADPH/NADP ratio. Similarly, the inferred (Fig. 2) and observed, low ratios of NADPH to NADP in G6PD-deficient cells indicate that several recently proposed regulatory mechanisms for the HMS in erythrocytes or other mammalian cells do not provide satisfactory explanations for the impaired intracellular function of the HMS in G6PD-deficient erythrocytes. Among these are the suggestions that the concentration of NADP is low (20, 21) or that the concentration of inhibiting NADPH is high (15, 22).

Sapag-Hagar, Lagunas, and Sols found, with rat liver cells, that the intracellular concentration of 6-phosphogluconate was low, despite a high ratio of G6PD/6-phosphogluconate dehydrogenase activity (23). They concluded, however, that the intracellular utilization of 6-phosphogluconate is greater than can be accounted for, rather than that intracellular generation of 6-phosphogluconate may be less than expected. The activity of the HMS would be underestimated if 6-phosphogluconate were also utilized by some enzyme other than 6-phosphogluconate dehydrogenase, as suggested by Sapag-Hagar *et al.* This possibility seems unlikely in human erythrocytes. We find that over 90% of the glucose consumed can be accounted for by $^{14}CO_2$ and ^{14}C-labeled anions, when methylene blue is added, yet the presence of methylene blue leads to no major increase in labeled anions (Table 2).

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