NADPH, Not Glutathione, Status Modulates Oxidant Sensitivity in Normal and Glucose-6-Phosphate Dehydrogenase-Deficient Erythrocytes

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Glucose-6-phosphate dehydrogenase (G6PD) deficiency is a common genetic disorder that can result in enhanced erythrocyte oxidant sensitivity and hemolytic anemia. G6PD deficiency results from a number of different mutations; however, the basic defect is a decreased or absent ability of the erythrocyte to generate reduced nicotinamide adenine dinucleotide (NADPH). Among the pleotropic effects of this deficiency is the inability of erythrocyte glutathione reductase, an NADPH-dependent enzyme, to cycle oxidized glutathione (GSSG) to reduced glutathione (GSH). The loss of NADPH and GSH are thought to account for the enhanced rates of methemoglobin generation, Heinz body formation, and lipid peroxidation that are observed in G6PD-deficient cells in response to both endogenous and exogenous oxidants.

Indeed, maintaining GSH in the reduced state has been thought to be the most important function of G6PD. In the absence of this enzyme, erythrocytes can have very low concentrations of GSH and, consequently, lack the cofactor necessary for normal glutathione peroxidase activity. This functional loss of glutathione peroxidase activity has been believed to be responsible for the enhanced sensitivity of G6PD-deficient red blood cells (RBCs) to H2O2-generating redox active drugs. The already-low concentration of GSH in G6PD-deficient cells is further exacerbated by the active extrusion of GSSG from the cell, further depleting the intracellular GSH pool.

However, several studies question whether GSH deficiency alone is sufficient to explain the oxidant sensitivity of G6PD-deficient cells. Low concentrations or the absence of GSH, by itself, appear to be insufficient to cause increased cellular susceptibility to H2O2 or H2O2-generating agents. In addition, the role of catalase in the detoxification of H2O2 may have been underestimated and may actually be of equal or greater importance than GSH in preventing H2O2-mediated injury. Nonetheless, as the increased oxidant sensitivity of G6PD-deficient erythrocytes is readily apparent, a mechanism for this effect must be found.

To further delineate the underlying causes of increased oxidant sensitivity in G6PD-deficient erythrocytes, an osmotic lysis and resealing technique has been used by which individual components of the erythrocyte can be discreetly manipulated while maintaining normal cellular characteristics. Using this technique, the roles of GSH, NADPH, and G6PD in cellular oxidant defense have been examined. These data indicate that decreased GSH concentration is not responsible for the increased oxidant sensitivity observed in G6PD deficiency. Rather, the enhanced oxidant sensitivity arises as a direct consequence of decreased NADPH concentration and is independent of the steady-state GSH status.

MATERIALS AND METHODS

Following informed consent, normal and G6PD-deficient erythrocytes were obtained in heparin from laboratory volunteers and outpatients at Children's Hospital, Oakland, CA. Blood samples were used within 24 hours of collection. Unless stated otherwise, all biochemicals were obtained from Sigma Chemical Co (St Louis, MO). Purified yeast G6PD (Sigma Cat. No. 4134) was used in the reconstitution of the G6PD-deficient erythrocytes.

RBC loading was conducted as previously described. Briefly, washed, packed RBCs (80% to 85% hematocrit) and G6PD (0 to 14.4 IU/ml packed RBCs), GSH (0 to 8 mmol/L final concentration), or NADPH (0 to 120 μmol/L final concentration) were mixed together in a test tube. The test tube contents were sealed in dialysis tubing (11.5-mm diameter; molecular weight cutoff of 3,500 daltons) as a thin film to maintain a high surface to volume ratio, thus insuring ready lysis and resealing. The samples were dialyzed against 1 L of lysis buffer (5 mmol/L potassium phosphate buffer,

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were assayed for their cell hemoglobin concentration (MCHC), and RBC distribution width (RDW, a measure of RBC density heterogeneity) on control and resealed erythrocytes were analyzed with a Coulter Counter (Coulter S Plus Counter; Coulter Electronics, Hialeah, FL). RBCs were assayed for G6PD activity as previously described and expressed as International Units per gram hemoglobin (IU/g Hb). GSH concentrations of the control and resealed RBCs were determined by the method of Beutler19 and expressed as micromoles per gram of Hb. NADP(H) values were determined by the method of Zerez et a19 and expressed as the ratio of NADPH to the total NADPH/NADP pool. Hemoglobin concentration was determined spectrophotometrically with ferricyanide-cyanide (Drabkin’s reagent) and read at 540 nm.31

To investigate whether enhanced intracellular GSH concentrations would affect cellular oxidant sensitivity, normal and G6PD-deficient erythrocytes were loaded with increasing concentrations of GSH. The effect of glutathione depletion on oxidant sensitivity was examined using 1-chloro-2,4-dinitrobenzene (CDNB)-treated erythrocytes and exposed to H2O2-generating systems. GSH depletion was accomplished by incubating control, resealed, and loaded erythrocytes (30% hematocrit) at 37°C for 1 hour in the presence of 1 mmol/L CDNB.22,23 Residual GSH concentration was then determined and found to be negligible.

The response of control, resealed, GSH-depleted/loaded, and G6PD-reconstituted RBCs to H2O2 generating systems was assessed by cellular lysis, methemoglobin formation, and GSH depletion. H2O2 was generated by 50 μmol/L phenazine methosulfate (PMS)24 or 50 μmol/L menadione sodium bisulfite.25,26 RBC lysis was determined by quantification of released Hb in the supernatant via ferricyanide-cyanide (Drabkin’s) reagent.27 Hemo-
globin oxidation was determined by spectrophotometric analysis (500 to 700 nm) and determining the concentration of oxyhemoglo-
bin, methemoglobin, and hemichrome hemoglobin according to the method of Winterbourn.27 and Szébeni et a128 GSH concentration was determined as described above.19

RESULTS

As previously shown,16,17 the osmotic lysis and resealing used in this study do not appreciably alter normal RBC characteristics. The MCV, MCHC, cellular deformability, adenosine triphosphate concentration, lipid composition, membrane permeability, and membrane transport mechanisms of RBCs are essentially unaffected by the resealing procedure.17 Similarly, erythrocyte oxidant sensitivity was also unaltered as a consequence of resealing.16,17 Analysis of the G6PD activity in the normal and G6PD-deficient cells showed activities of 7.66 ± 0.65 and 0.18 ± 0.19 IU/g Hb, respectively. As was expected, the GSH concentration in the control-resealed G6PD-deficient cells was substantially reduced (1.92 ± 0.70 μmol/g Hb) in comparison with control-resealed normal erythrocytes (6.84 ± 0.93 μmol/g Hb).

The oxidant sensitivity of normal and G6PD-deficient cells to H2O2-generating agents was found to be distinctly different. As shown in Fig 1, G6PD-deficient cells were significantly more sensitive to PMS. Similar results were observed after menadione challenge (data not shown). Concurrent with the increase in methemoglobin was a rapid loss of cellular GSH (Fig 2). While normal cells maintained approximately 70% of their initial GSH concentration following PMS challenge, the G6PD-deficient cells demonstrated a total depletion of cellular GSH. These results were entirely consistent with an important role for GSH in the enhanced oxidant sensitivity of G6PD-deficient cells.

However, direct examination of the role of GSH, by depletion and supplementation studies, in normal and G6PD-deficient cells demonstrated that oxidant sensitivity was independent of GSH concentration. As shown in Fig 1, depletion of erythrocyte GSH by CDNB had no adverse effect on methemoglobin generation in either the normal or enzyme-deficient cells. Similarly, loading of erythrocytes with even high levels of GSH (> 20 μmol/g Hb) had no beneficial effect of erythrocyte oxidant sensitivity (Fig 1) even though these high levels were maintained throughout the course of the experiment in the normal erythrocytes. Similarly, GSH depletion or supplementation of cellular GSH had no effect on erythrocyte lysis (Fig 3).

Conversely, entrapment of a NADPH-generating enzyme (purified G6PD) within the G6PD-deficient cells resulted in significantly decreased sensitivity to H2O2-generating agents (Fig 4). The extent of protection was...
dependent on the entrapped enzyme activity. However, concurrent with NADPH generation came an ability to maintain intracellular GSH concentrations in response to oxidant challenge (Fig 3). Although this latter effect resulted in maintenance of only a small steady-state GSH concentration, this could potentially explain the protective effects of these enzymes. To determine whether maintenance of the cellular GSH pool was responsible for the increased oxidant resistance, the G6PD-loaded cells were depleted of GSH by CDNB treatment. As shown in Fig 4, GSH depletion did not diminish or abolish the enhanced oxidant resistance of the G6PD-loaded cells.

Further examination of the relationship between methemoglobin generation and GSH or NADPH concentration definitively showed a strong inverse correlation between NADPH levels and methemoglobin concentration ($R^2 = .854$; Fig 5A). No correlation between GSH concentration and methemoglobin was observed in either the normal ($R^2 = .029$) or G6PD-deficient ($R^2 = .041$) erythrocytes (Fig 5B).

**DISCUSSION**

As a consequence of their physiologic role, erythrocytes are exposed to continuous oxidant stress. This has necessitated the coevolution of enzymatic pathways by which these reactive oxygen species are detoxified. Hydrogen peroxide, which is produced in the course of normal cellular events, is catabolized by either the GSH-dependent action of glutathione peroxidase or by catalase. High enzymatic activities of both of these systems are present in the normal RBC. Nonetheless, the GSH-dependent activity of glutathione peroxidase has been generally viewed as the primary defense against $\text{H}_2\text{O}_2$ in the erythrocyte.

G6PD deficiency is the most common inherited enzyme abnormality in humans and is characterized by increased sensitivity to $\text{H}_2\text{O}_2$-generating agents. The enhanced oxidant sensitivity of G6PD-deficient erythrocytes has been thought to arise as an indirect consequence of NADPH depletion, which resulted in a decreased or absent ability to cycle GSH, thus conferring the oxidant sensitivity.
NADPH-generating system restored substantial oxidant resistance in the G6PD-deficient cells. Analysis of methemoglobin generation and intracellular GSH concentration clearly demonstrated that no correlation existed between intracellular GSH status and Hb oxidant sensitivity in either normal or G6PD-deficient erythrocytes. Thus, as demonstrated in this study and others, \(^{11,15-17}\) loss of GSH alone does not appear to be sufficient to explain the elevated \(\text{H}_2\text{O}_2\) susceptibility noted in G6PD deficiency.

In contrast, as shown in this study and others, \(^{15,34}\) an NADPH-generating capacity appears to be crucial for resistance to \(\text{H}_2\text{O}_2\)-generating agents. Hence, an alternative mechanism must be evoked to explain the readily apparent oxidant sensitivity of G6PD-deficient erythrocytes. Several recent studies have implicated an important role for NADPH in not only sustaining GSH levels but also in maintaining the catalytic activity of catalase. \(^{15,34,35}\) As a result, NADPH appears to be essential for the catalytic activity of both major \(\text{H}_2\text{O}_2\) catabolizing pathways. Hence, G6PD-deficient individuals may functionally lack both glutathione peroxidase and catalase activity. In the absence of only one of these systems (eg, GSH depletion or acatalasemia), erythrocytes exposed to normal in vivo fluxes of \(\text{H}_2\text{O}_2\) might readily survive, whereas decreased or absent activity of both defense systems might accompany insufficient NADPH in G6PD deficiency and may make cells susceptible to even the low levels of \(\text{H}_2\text{O}_2\) generated in vivo or to exogenously administered redox active compounds such as primaquine and divicine. Reconstitution of G6PD-deficient cells, as shown here and in other studies, \(^{36-38}\) results in increased resistance to exogenous oxidants. However, this resistance is independent of GSH.

While normal erythrocytes have the capacity to reduce methemoglobin back to the ferrous state via the NADH-dependent action of methemoglobin reductase (NADH-cytochrome b5 reductase), \(^{39,40}\) it is also possible that NADPH may also play an important role in reducing methemoglobin to oxyhemoglobin. \(^{41,44}\) The results shown in this study indicate that normal erythrocytes can reduce the level of methemoglobin following PMS challenge (Fig 1). In contrast, G6PD-deficient cells do not have this capacity (Fig 1). However, entrapment of G6PD in the deficient cells restores apparent methemoglobin reductase activity (Fig 4). Taken together, these data suggest that NADPH concentration may be of importance in preventing methemoglobin generation and/or accumulation. Indeed, an NADPH-flavin reductase pathway for methemoglobin reduction has been previously described in the erythrocyte and may account for up to 20% of methemoglobin reduction under normal conditions. \(^{42,43}\) The NADPH-dependent pathway may be of even more importance in pathologic cells that contain elevated Ca\(^{2+}\) levels because it is activated by Ca\(^{2+}\), whereas the NADH-dependent reductase is significantly inhibited. \(^{44}\)

In conclusion, the enhanced oxidant sensitivity of G6PD-deficient cells is not due to reduced intracellular GSH; rather, it is most likely due to the absence of NADPH and the functional impairment of both GSH/glutathione peroxi-
dase and catalase-mediated catabolism of H$_2$O$_2$. This is not to imply that GSH is without function in the erythrocyte. Indeed, GSH may be of great importance in protecting against membrane protein oxidation and lipid oxidation, chelation of free heme, and the GSH/GSSG ratio may be of significance in the metabolic regulation of the cell. Alterations of these functions would further exacerbate the oxidant sensitivity of G6PD-deficient cells. However, the role of NADPH in cellular oxidant defense must be reexamined. Indeed, altered NADPH status may be of importance in other erythrocyte abnormalities, such as pyruvate-kinase deficiency, B-thalassemia, and sickle cell anemia, which are characterized by enhanced oxidant sensitivity and impaired NAD synthesis and or low levels of NADPH.

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