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Authors

Chang, Jae C

van der Hoeven, Ludolph H

Haddox, Charles H

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Glutathione Reductase in the Red Blood Cells

JAE C. CHANG, M.D., LUDOLPH H. VAN DER HOEVEN, M.D.,
and CHARLES H. HADDOX, Ph.D.

*Department of Internal Medicine
and Department of Laboratory Medicine,
Good Samaritan Hospital and Health Center,
Dayton, OH 45406*

*and
Department of Medicine and Department of Pathology,
Wright State University School of Medicine,
Dayton, OH 45406*

ABSTRACT

Glutathione reductase plays an important role in protecting hemoglobin, red cell enzymes, and biological cell membranes against oxidative damage by increasing the level of reduced glutathione (GSSG) in the process of aerobic glycolysis. The enzyme deficiency may result in mild to moderately severe hemolytic anemia upon exposure to certain drugs or chemicals. However, hereditary deficiency of the enzyme is extremely rare. Recent studies on glutathione reductase in the red cell have shown more insight in the understanding of red cell metabolism and interactions with other enzymes, especially glucose-6-phosphate dehydrogenase (G-6-PD).

Glutathione reductase in serum may be a source of error in any clinical laboratory test in which an enzyme activity is determined indirectly by measuring the change in reduced nicotinamide-adenine dinucleotide (NADH) or reduced nicotinamide adenine dinucleotide phosphate (NADPH) absorbance. Glutathione reductase levels are reduced in banked blood when citrate-phosphate-dextrose (CPD) is used as a preservative. Reviewed is the role of glutathione reductase in the metabolism of the red cell and its clinical implication and usefulness.

Introduction

The history of glutathione reductase, an important red cell enzyme acting in aerobic glycolysis of red cell metabolism, dates back to 1931, when Hopkins and Elliott demonstrated in livers of various animals a heat-labile system which was

capable of reducing oxidized glutathione.¹⁰ Soon after, Meldrum and Tarr confirmed the ability of erythrocytes to catalyze glutathione reduction.¹⁷

Then, in 1955, Racker isolated glutathione reductase in crystalline form from yeast for the first time.²² Glutathione

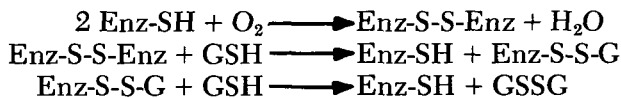
reductase has been found not only in many animal tissues, but also in microorganisms, yeasts and higher plants.¹⁸ Recently, there have been numerous studies elucidating the enzymatic and metabolic interactions of glutathione reductase in human erythrocytes and other mammalian tissues.

Glutathione reductase (EC 1.6.4.2) (GR) is a flavin enzyme which reduces oxidized glutathione (GSSG) to reduced glutathione (GSH) with the concomitant oxidation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) to nicotinamide adenine dinucleotide phosphate (NADP), thus:



As seen in this equation, its main function is maintenance of glutathione in a reduced state.

Reduced glutathione is a cellular protein containing sulfhydryl groups and is responsible for the stability of many red cell enzymes, hemoglobin and certain structural proteins in red cell membrane. In the normal physiologic process, spontaneous oxidation of these enzymes and other proteins may lead to disulfide formation. Consecutive disulfide exchange reactions with reduced glutathione can serve to restore the active sulfhydryl forms, thus:



The oxidized glutathione then can be reduced by glutathione reductase allowing continuous repetition of the cycle. Further, reduced glutathione functions as a specific coenzyme for glyoxalase activity; in this reaction, methylglyoxal undergoes as intramolecular oxidation-reduction to lactate.

Glutathione reductase may also be regarded as a regulator of the proper bal-

ance of NADP and NADPH in the hexose monophosphate shunt of glycolysis (figure 1).

Principle of Glutathione Reductase Determination

Glutathione reductase is one of two red cell enzymes requiring riboflavin for its activation.^{2,8} Recent investigations have shown that erythrocyte glutathione is present in at least two forms, an active form associated with flavin adenine dinucleotide (FAD) and an inactive form not bound to FAD, which can be activated *in vitro* by additional extraneous FAD.^{3,12} The FAD is derived from ribo-

flavin via flavin mononucleotide (FMN). *In vitro*, neither riboflavin nor FMN has any effect on glutathione reductase activity; however, *in vivo* administration of riboflavin results in a marked stimulation of red cell glutathione reductase activity. It has been demonstrated that measurement of both active and inactive forms of glutathione reductase may have a clinical value in the assessment of nutritional status of riboflavin.^{7,8,25,28}

Hemolysate containing glutathione reductase is added to the mixture of GSSG and NADPH. In this reaction, the degree of oxidation of NADPH to NADP repre-

sents the activity of glutathione reductase and the enzyme activity is measured by the changes in absorbance in a UV Spectrophotometer at 340 nm. A measurement obtained in this way represents the activity of the enzyme complex FAD-GR or the *in vivo* active form of glutathione reductase. In order to measure total glutathione reductase activity, which includes the inactive form, FAD must be added *in*

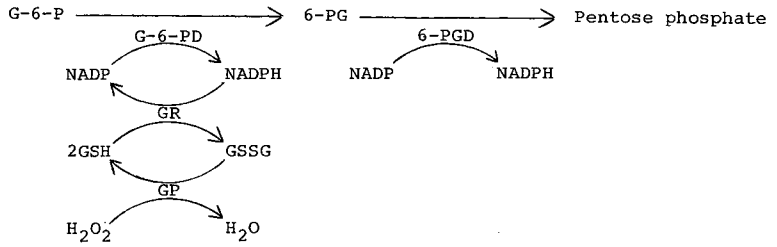


FIGURE 1. Pathway for glutathione metabolism in the red cell.

G-6-P = Glucose-6-phosphate; 6-PG = 6-phosphogluconate; R-5-P = ribose-5-phosphate; G-6-PD = glucose-6-phosphate dehydrogenase; 6-PGD = 6-phosphogluconate dehydrogenase; NADP = oxidized nicotinamide adenine dinucleotide phosphate; NADPH = reduced nicotinamide adenine dinucleotide phosphate; GSH = reduced glutathione; GSSG = oxidized glutathione; GR = glutathione reductase; GP = glutathione peroxidase.

vitro (figure 2). In our laboratory, the normal range of total glutathione reductase activity is 5.65 ± 0.82 (2 S.D.) units per g of Hb or 1.55 ± 0.24 (2 S.D.) IU/10¹⁰ red blood cells (RBC). The active fraction normally exhibits less than 75 percent of the total glutathione reductase activity.⁹

Interference by Glutathione Reductase in Other Enzyme Determinations

Glutathione reductase present in serum may be a source of error in any clinical laboratory test in which an enzyme activity is determined indirectly by measuring the change in NADH or NADPH absorbance. Some glutathione reductase activity is found in normal serum, and above normal amounts have

been reported in the sera of patients with liver diseases and certain anemias.²⁶ The possible source of error may be due to the following properties of glutathione reductase:

1. The optimal substrate concentration is 2 m mol of GSSG per liter, but the enzyme activity may be considerable with concentrations as low as 0.2 m mol per liter.
2. The pH curve is relatively flat with a broad maximum between 6.4 and 7.4.
3. The coenzyme NADH may function almost as efficiently as NADPH. At sub-optimal substrate concentrations, the reaction rates are almost identical.
4. Glutathione reductase is very stable. A 60 minute incubation at 56° does not result in loss of activity.

Reactants

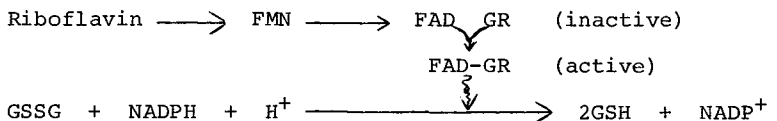
Substrate: Glutathione

Co-enzyme: NADPH

Enzyme activator: FAD (flavin adenine dinucleotide)

FIGURE 2. Assay of glutathione reductase.

Principle



FMN = Flavin mononucleotide

Reduced glutathione is used as a stabilizer in many commercial test kits. It may or may not be listed as present. If the reagents in the kit have been lyophilized, a considerable proportion of the glutathione will be oxidized during this process. This may result in a number of problems. For example, a creatine kinase (ED 2.7.3.2) kit contains glutathione at 28 m Molar (lyophilized reagent mixture). An appreciable amount of the glutathione should be in the oxidized form, certainly more than the 0.2 m Molar concentration mentioned. NADH or NADPH formed during the measurement of creatine kinase is reoxidized by glutathione reductase and this concomitant reaction results in falsely low values for creatine kinase. Sera with high glutathione reductase activity can obscure a low normal creatine kinase activity and present a reverse reaction.¹⁴ Wiedemann found that 2 percent of a selected population showed falsely low creatine kinase values owing to glutathione reductase.³⁰

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities are determined indirectly by measuring the decrease in NADH absorbance. If oxidized glutathione is present in the reaction mixture, serum glutathione reductase will result in additional loss of NADH. Consequently, aspartate aminotransferase and alanine aminotransferase values will be falsely elevated. Klotzsch examined several commercial kits for determination of aspartate aminotransferase and alanine aminotransferase.¹³ Some brands of lyophilized reagent mixtures contained oxidized glutathione at 1.0 to 1.5 m Molar. A difference of 5 to 10 percent in enzyme activity was found when compared with reagents of the same composition but without glutathione.

Glutathione Reductase Levels in Stored Blood

Glutathione reductase levels were measured in aliquots removed from dis-

carded units of blood received from the Community Blood Center. All units were 6 to 8 days old. All units contained the CPD* preservative as recommended by the American Association of Blood Banks.¹⁹ Glutathione reductase levels in 18 units were 0.80 to 1.13 IU/10¹⁰ RBC. These values are roughly 15 to 40 percent below the lower limit of our normal (1.31 IU/10¹⁰ RBC). Aliquots removed weekly during the following four weeks did not show any significant changes in the levels from each unit. Blood samples drawn when the blood was collected were unavailable.

Blood samples were drawn from laboratory personnel and placed immediately in either heparin or in CPD (at concentrations comparable to that in the standard blood bank unit). The glutathione reductase levels with CPD were 7 to 9 percent lower than with heparin. After a storage period of 24 hours, the glutathione reductase level in the CPD preserved blood was 17 to 27 percent lower than those containing heparin. The glutathione reductase levels were essentially unchanged in the heparin samples after 24 hours.

Clinical Use of Glutathione Reductase

HEREDITARY GLUTATHIONE REDUCTASE DEFICIENCY

The true glutathione reductase deficiency of genetic type is rare. Nevertheless, several cases have been well studied.^{21,27,29} The inherited defect appears to be an autosomal dominant trait and the enzyme activity in hereditary glutathione reductase deficiency is about one-half of the normal value.²⁹ Interestingly, other red cell enzymes involved in glycolysis, especially G-6-PD, tend to be increased in these individuals, presumably due to an increased number of young red cells in the presence of hemolysis.

* Citrate phosphate dextrose.

Hematologic findings include normo- to macrocytic nonspherocytic hemolytic anemia when exposed to certain drugs. The anemia may be associated with thrombo- and granulocytopenia and about one-fourth of the patients develop pancytopenia after drug exposure.²⁹

The chemical composition of the drugs that are known to induce hemolytic anemia or pancytopenia on the basis of glutathione reductase is similar to or identical with that of drugs inducing hemolysis in G-6-PD deficiency. Drugs which have been associated with hemolysis or pancytopenia include azathioprin, azulfidine, dapsone, chloramphenicol, primaquine, chloroquine, acetylsalicylic acid, acetophenetidin, phenylbutazone, pyramidone and phenprocoumon. Heinz bodies may be demonstrated in red cells of patients with brisk hemolysis after drug exposure. Anemia is mild to moderate in severity and evidence of hemolysis is usually present. When there is a chronic hemolytic anemia, splenectomy may be beneficial in reducing hemolytic process. Some patients with glutathione reductase deficiency may show abnormal neurologic findings such as oligophrenia or spastic neurologic signs.²⁹

Although hereditary glutathione deficiency is extremely rare, a low level of glutathione reductase owing to metabolic disturbances may not be uncommon. Frischer et al studied the prevalence of glutathione reductase in 3,159 apparently healthy, unrelated individuals from the United States, South Vietnam, Iran and Ethiopia.⁶ A decreased level of the enzyme was found in 0.3 percent of European-Americans, 1.9 percent of Afro-Americans, 7.3 percent of Ethiopians, 14.6 percent of Iranians and 22.0 percent of South Vietnamese. Therefore, the possibility of an environmental factor such as riboflavin deficiency was suggested as a frequent cause of decreased activity of glutathione reductase.

DISORDERS WITH HIGH GLUTATHIONE REDUCTASE

Glutathione reductase deficiency results in an increase of the activity of G-6-PD and vice versa. The mechanism of increased activity of glutathione reductase in G-6-PD deficiency is not well understood; however, it is presumably due to the release of glutathione reductase from inhibition secondary to decreased production of phosphogluconolactone and 6-phosphogluconate with accumulation of NADP.⁶ In addition to the elevation of total glutathione reductase, an increased level of the active form of glutathione reductase, FAD-GR, was found in G-6-PD deficiency. The explanation is that G-6-PD deficiency stresses the hexose monophosphate shunt and results in compensatory activation of glutathione reductase by the increase of FAD binding.

Glutathione reductase was also found to be increased in patients with liver cirrhosis and uremia.³² Increased saturation of glutathione reductase with FAD was again noted in these conditions and it was suggested that the increased requirements of an enhanced activity of the hexose monophosphate shunt may affect glutathione metabolism secondarily, leading to the binding of glutathione reductase with FAD through the mechanism of increased uptake of riboflavin.

Other conditions known to cause the increased glutathione reductase activity are malignant disease,^{5,31} diabetes,¹⁵ cystic fibrosis^{23,24} and administration of nicotinic acid.^{4,16}

VALUE OF GR IN EVALUATING RIBOFLAVIN NUTRITIONAL STATUS

A constant supply of riboflavin is required in man for the optimal maintenance of many metabolic processes requiring the flavin coenzyme. The level of red cell and urinary riboflavin is easily influenced by daily intake of riboflavin, and its measurement is not satisfactory in

evaluating riboflavin nutritional status. However, protein-bound riboflavin is relatively stable and not markedly influenced by oral dietary riboflavin intakes. Thus, a preferred assay for riboflavin nutritional status would be direct measurement of protein-bound riboflavin.⁸

Glutathione reductase is one of two flavoproteins in erythrocytes requiring FAD,² and it has been suggested that its activity reflects the nutritional status for riboflavin.^{7,8} Glatzle et al measured the activity of glutathione reductase *in vitro* with and without FAD, expressing their results as the degree of stimulation or activation coefficient (AC).⁷

$$AC = \frac{\text{Reduction of absorbance with added FAD/10 min}}{\text{Reduction of absorbance without FAD/10 min}}$$

In studies of more than 300 persons, activation coefficient ≥ 1.20 was well correlated with an evidence of biochemical riboflavin deficiency; whereas values < 1.20 were considered to be within normal range. Since then other investigators have confirmed the value of activated glutathione reductase determination in assessing riboflavin nutritional status.^{1,11,20,25,28}

These findings suggest the determination of glutathione reductase activation is a useful test in evaluating suspicious cases of riboflavin deficiency and assessing riboflavin nutritional status.

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