The Response of Red Cell Hexose Monophosphate Shunt After Sulfhydryl Inhibition

By Arthur L. Sagone, Jr., Stanley P. Balcerzak, and Earl N. Metz

In this investigation, we studied the importance of cellular glutathione (GSH) in the hexose monophosphate shunt (HMPS) activity of unstimulated human erythrocytes and the mechanism by which pyruvate stimulates the HMPS. The rate of HMPS activity was measured by the production of radioactive CO₂ from ¹⁴C-1-glucose or ¹⁴C-1-ribose using a vibrating reed electrometer and ionization chamber. HMPS activity was not significantly impaired by N-ethylmaleimide (NEM) in concentrations which bound all red cell GSH. Red cells incubated under carbon monoxide (CO), an experimental condition which eliminates peroxide production, still had HMPS activity which was 44% of the value under air. Pyruvate stimulation of the HMPS was unaffected by doses of NEM which bound all cellular GSH or by incubation under CO. These data indicated that pyruvate stimulation of the HMPS occurs by pathways which do not involve peroxide formation, GSH, or oxygen. This study indicates that sulfhydryl blockade of GSH does not necessarily inhibit HMPS activity and that HMPS activity in red cells may respond to reactions not linked directly to glutathione reduction.

In spite of significant differences in experimental methods, several investigators have reported similar rates of hexose monophosphate shunt activity (HMPS) in human erythrocytes.¹⁻⁶ According to present theory, the activity is governed primarily by glutathione metabolism.⁷⁻⁸ In this scheme, oxidized glutathione (GSSG) is formed from reduced glutathione (GSH) by the enzyme glutathione peroxidase. Reduction of GSSG then occurs by the enzyme glutathione reductase with simultaneous oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) to oxidized nicotinamide adenine dinucleotide phosphate (NADP). Finally, NADPH is regenerated by the oxidative portion of the hexose monophosphate shunt.⁷

Although the role of glutathione in the stimulation of HMPS by agents which generate cellular peroxide is well established,⁷⁻⁸ the role of GSH in unstimulated HMPS activity is less clear. The possibility that part or all of this HMPS activity of red cells is unrelated to glutathione metabolism or that significant stimulation of the HMPS may occur by nonglutathione pathways has not been extensively studied.

The present study examined the importance of cellular glutathione in the HMPS activity of unstimulated red cells. The rate of HMPS activity was measured by the production of CO₂ from ¹⁴C-1-glucose substrate in the presence of N-ethylmaleimide (NEM), an agent which binds GSH, and in the presence...
of carbon monoxide, an experimental condition which eliminates the production of cellular peroxide. Also, the mechanism of pyruvate stimulation of the HMPS was studied to determine if a physiologic compound could alter HMPS activity by a pathway unrelated to glutathione metabolism or peroxide formation.

MATERIALS AND METHODS

Venous blood was collected in heparinized tubes from normal volunteers and centrifuged at 1000 g for 10 min. The plasma and buffy coat were removed and the red cells were washed once in 5 volumes of saline and resuspended in a pH 7.4 buffer containing 145 mM Na⁺, 5 mM K⁺, 20 mM glycylglycine, 5 mM glucose, and 145 mM Cl⁻. White cell and platelet contamination was less than 1000 cu mm and 10,000/cu mm, respectively. Three to four milliliters of red cells were suspended in a final volume of 10 ml in a 50-ml triple-headed distilling flask to which was then added 5 μCi of radioactive glucose or ribose labeled in the first position. (All radioactive reagents obtained from Amersham-Seale Corp., Arlington Heights, Ill.) In experiments with labeled ribose, glucose was omitted from the medium and replaced by 5 mM ribose.

The inlet of the flask was connected to a gas cylinder containing either compressed air or carbon monoxide. The outlet arm of the flask was connected to a 275-ml Cary-Tolbert ionization chamber and a Cary Model 401 vibrating reed electrometer (Cary Instruments, Monrovia, Calif.) The third arm of the flask was covered with a rubber stopper through which reagents could be added or samples withdrawn through a spinal needle. The use of the ionization chamber-electrometer apparatus for continuous monitoring of CO₂ produced by cell suspensions and our modifications of this apparatus have been described in detail elsewhere. A duplicate system was used so that CO₂ derived from ¹⁴C-l-glucose should be measured simultaneously from both control and the experimental flasks. The incubation flasks were maintained at 37°C throughout these experiments and were stirred continuously. After base-line CO₂ production was established, agents (in buffer) were added through the center well to the experimental flasks and an equal volume of buffer alone (0.1-0.3 cc) to the control flasks. CO₂ production was calculated from the millivolt reading recorded once steady conditions were re-established in the experimental chamber and compared to the corresponding value from the control flask. Total CO₂ production was calculated as previously described and expressed in micromoles CO₂ per milliliter RBC per hour.

NEM was added in amounts ranging from 2 to 4 μmoles/ml of packed red cells. Sodium pyruvate was added in a final concentration of 1 or 10 mM. Methylene blue was added in final concentration of 10⁻⁶ M.

Red cell GSH values were determined using 5,5'-dithiobis(-2 nitrobenzoic acid) according to the method of Beutler, Duron, and Kelly. Glucose concentrations were measured by the glucose oxidase method.

RESULTS

Effect of NEM on CO₂ Production and Glucose Consumption

The addition of 2 μmoles of NEM per ml of packed red cells resulted in an increased CO₂ production from ¹⁴C-l-glucose of 0.106 μmoles/ml RBC/hr compared to the control value of 0.069 (see Fig. 1, p < 0.001). This amount of NEM was enough to bind 80%-90% of the intracellular GSH, since red cell GSH levels ranged from 2.25 to 2.50 μmoles/ml of packed cells. CO₂ production from ¹⁴C-l-glucose in the presence of 3 μmoles of NEM, a dose slightly in excess of that required to bind all cellular GSH, was not significantly different from control suspensions. Four micromoles of NEM per milliliter, a dose in significant excess of that required to bind red cell GSH, resulted in only a 25% decrease in CO₂ production from ¹⁴C-l-glucose of 0.052 μmoles/hr/ml RBC (p < 0.001, Fig. 1). No GSH could be detected in red cells in the presence of either the 3- or 4-μmole doses of NEM.
Mean glucose consumptions of suspensions in the presence of 2 and 3 μmoles of NEM were 0.82 and 0.75 μmole/ml RBC/hr, respectively, and not significantly different from the control value of 0.85 μmole/ml RBC/hr (Fig. 1). The mean glucose consumptions of red cell suspensions in the presence of 4 μmoles of NEM was 0.62 μmole/ml/hr and mildly reduced compared to control suspensions (p < 0.05). This reduction in glucose consumption of 28% was similar to the reduction in CO₂ production from ¹⁴C-1-glucose of 25% in the presence of 4 μmoles of NEM and suggested that the apparent decrease of HMPS activity in the presence of 4 μmoles NEM was the result of decreased hexokinase activity rather than a direct effect on the oxidative portion of the HMPS.

To test this suggestion, CO₂ production from ¹⁴C-1-ribose was used to measure HMPS activity in the presence of NEM. This substrate does not require hexokinase to be metabolized by HMPS of red cells. As indicated in Fig. 2, CO₂ production from ¹⁴C-1-ribose in the presence of 2 μmoles of NEM was increased compared to controls, similar to experiments in which ¹⁴C-1-glucose was used as a substrate. In the presence of 4 μmoles of NEM, CO₂ from ¹⁴C-1-ribose was further increased to 0.067 μmole/ml/hr and was in contrast to the reduced CO₂ from ¹⁴C-1-glucose in the presence of 4 μmoles NEM.

![Fig. 1. Effect of NEM on CO₂ production and glucose consumption. Mean CO₂ production from ¹⁴C-1-glucose for controls and suspensions incubated with 2 and 4 μmoles of NEM are indicated by the open bar (six experiments). Corresponding mean glucose consumptions are indicated by the hatched bars (five experiments). Results with 3 μmoles were similar to controls and are not shown. Standard deviations are indicated by the brackets.](image1)

![Fig. 2. Effect of NEM on CO₂ production from ribose. This graph is typical of five experiments measuring CO₂ production from ¹⁴C-1-ribose using an ionization chamber-electrometer apparatus. Controls are indicated by dashed lines, experimental by solid lines. After base-line CO₂ production was established, NEM was added as indicated.](image2)
Effect of Carbon Monoxide on CO₂ Production

In the presence of carbon monoxide, CO₂ production from ¹⁴C-l-glucose was reduced to \((0.036 \pm 0.009 \mu\text{mole/ml/hr})\) compared to a mean value of \((0.081 \pm 0.026)\) in suspensions incubated in air. This difference, based on seven paired experiments, is highly significant \((p < 0.001)\). The addition of NEM in amounts which bound all intracellular GSH but did not alter RBC glucose consumption \((3 \mu\text{moles NEM/ml RBC})\) had no effect on this rate of ¹⁴CO₂ production under CO.

Effect of Pyruvate on CO₂ Production

Ten millimolar pyruvate resulted in a three-fold increase in CO₂ production from ¹⁴C-l-glucose over base-line values. A typical experiment is represented by Fig. 3. No increase in CO₂ production occurred when ¹⁴C-6-glucose was used as a substrate, indicating that the increased CO₂ from ¹⁴C-1-glucose was actually the result of increased HMPS activity and not from residual enzymes of the Krebs cycle. Similarly, a twofold stimulation in CO₂ production could be demonstrated in the presence of 1 mM pyruvate. The addition of NEM \((3 \mu\text{moles NEM/ml RBC})\) had no effect on this pyruvate stimulation of the HMPS whether added before or after the pyruvate. As seen in Fig. 3, pyruvate stimulation of CO₂ production also occurred in the presence of CO.

The failure of methylene blue \((10^{-6} M)\) to stimulate CO₂ production in red cells incubated under CO confirms that the system was anaerobic (Fig. 3). As seen in Fig. 3, methylene blue stimulation of CO₂ production in air was not affected by prior incubation with pyruvate.

DISCUSSION

Current concepts linking HMPS activity to glutathione metabolism would predict that HMPS activity should not occur in the absence of GSH or cellular peroxide formation. The present study, however, shows that HMPS activity still occurs in the presence of concentrations of NEM which bind all intracellular GSH and under conditions in which no peroxide is generated. In experiments using ¹⁴C-l-glucose as a substrate, no alteration in HMPS activity...
could be demonstrated in the presence of 3 μmoles of NEM, an amount in slight excess of that required to bind all GSH, and only a 25% reduction in HMPS activity with 4 μmoles of NEM, an amount in significant excess of that required to block GSH.

The apparent reduction in HMPS activity with 14C-1-glucose substrate in the presence of 4 μmoles of NEM is consistent with impaired hexokinase activity, as indicated by the findings of decreased red cell glucose consumption and increased HMPS activity when ribose is used as a substrate. Since this pentose sugar is metabolized by the oxidative portion of the HMPS as a result of feedback through the enzymes transketolase and transaldolase (nonoxidative portion), it does not require hexokinase activity.1,6 These data are similar to that reported by Eldjarn et al. in red cells13 and other tissues14 and indicate that the enzymes of the oxidative portion of the HMPS are not adversely affected by sulfhydryl blockade. Further, our data with NEM raise the possibility that some or all of hexose monophosphate activity in unstimulated red cells is unrelated to GSH metabolism. Current knowledge regarding the HMPS indicates that stimulation of this pathway must be related ultimately to an increased ratio of NADP+ to NADPH, but physiologic reactions responsible for this, apart from those involving GSH, remain to be identified.

Our experiments with carbon monoxide indicate that part of the HMPS of unstimulated red cell suspensions is unrelated to cellular peroxide formation. Although incubation of red cell suspensions under carbon monoxide was associated with reduction in CO2 production from 14C-1-glucose, HMPS activity was still present and was 44% of the activity measured under air. These data indicate that part of HMPS activity of unstimulated red cell suspensions is related to reactions not requiring oxygen and is significantly higher than the value of 20% reported by Szeinberg and Marks.3 Since we have previously demonstrated that peroxide generation does not occur under carbon monoxide,15 this anaerobic HMPS activity cannot be related to glutathione peroxidase. Further, the failure of NEM to alter this activity indicates that it is not related to an alternate enzyme pathway involving glutathione. Whether the remaining HMPS activity is directly related to glutathione metabolism or other cellular reactions requiring oxygen is not clear from our results.

Our experiments indicate that pyruvate stimulates HMPS activity, and our observations are similar to those reported by other investigators.2 In addition, our studies indicate that the mechanism of this stimulation is unrelated to glutathione because 3 μmoles NEM, a dose sufficient to bind all red cell GSH, does not inhibit the response. Further, pyruvate stimulation was unaffected by carbon monoxide, indicating that the reaction does not require the presence of oxygen or peroxide formation. These observations are relevant to normal red cell metabolism. Both our studies and those of Szeinberg and Marks2 indicate the stimulation of the HMPS by pyruvate can occur in concentrations as low as 1 mM, a concentration of pyruvate which may occur in serum in several clinical conditions.16,17 This supports further the possibility that metabolic pathways unrelated to GSH may have significant importance in the normal regulation of HMPS activity in vivo. The mechanism of pyruvate stimulation of the HMPS is not clear. Several investigators have attempted without success to link pyruvate stimulation of the HMPS to enhanced activity of lactic acid de-
hydrogenase (LDH) with NADPH as a cofactor. Using red cell hemolysates, Szeinberg concluded that NADPH could not serve as a significant cofactor for LDH over a pH of 6.0-7.4. Karnovsky reported similar results in the study of granulocyte LDH. Recent studies by Beutler suggest that pyruvate stimulation of the HMPS might be related to LDH at low pH (6.9) but cannot account for the stimulation of the HMPS by pyruvate at pH 7.4. Similarly, attempts to link pyruvate stimulation of HMPS to a transhydrogenase reaction involving NADPH and NADH have also been unsuccessful. Our attempt to link pyruvate stimulation of the HMPS to GSH metabolism was also unsuccessful. Finally, our studies with CO and pyruvate raise the possibility that pathways which do not require oxygen are important in the metabolism of the HMPS of red cells. Further studies of these non-glutathione pathways in erythrocytes are required to determine their biologic importance.

ACKNOWLEDGMENT

The authors are indebted to Miss Rosemarie Husney for excellent technical assistance throughout these studies.

REFERENCES

The response of red cell hexose monophosphate shunt after sulfhydryl inhibition

AL Jr Sagone, SP Balcerzak and EN Metz