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ROLE OF HEXOKINASE IN THE REGULATION OF ERYTHROCYTE HEXOSE MONOPHOSPHATE PATHWAY UNDER OXIDATIVE STRESS

Mauro Magnani, Luigia Rossi, Marzia Bianchi
Giordano Serafini and Vilberto Stocchi

Istituto di Chimica Biologica, Università degli Studi, 61029 Urbino, Italy

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SUMMARY: Human erythrocytes overloaded with homogeneous human hexokinase (up to 15-times the activity of normal RBC) show almost unmodified rates of glucose metabolized in the HMP, however hexokinase-loaded RBC are able to metabolize 1.5 fold more glucose than controls through the HMP when an oxidizing agent like methylene blue (5 to 100 μM) is present. Similarly, RBC loaded with inactivating anti-hexokinase IgG (12 $\pm$ 3% residual hexokinase activity) show HMP rates unchanged under resting conditions, but only 12% of the HMP rate found in normal controls under oxidative stress. These data provide clear evidence that the HMP rate under conditions of oxidative stress is controlled by hexokinase activity and suggest that RBC from patients with hexokinase deficiency are not able to increase the HMP rate under oxidative stress like erythrocytes from individuals with G6PD deficiency.

In erythrocytes the HMP provides reducing equivalents (NADPH) for the maintenance of glutathione in reduced form as an anti-oxidative mechanism.

It is generally agreed that the regulation of erythrocyte HMP occurs by control of G6PD activity through inhibition by its dinucleotide product, NADPH (1-6) and by binding of coenzymes by intracellular proteins other than the enzyme itself (7). In contrast, under condition of oxidative stress (i.e. by addition of methylene blue), it has been suggested that HMP could be limited by G6PD (5) or by hexokinase (8-10), however the validity of these and other studies has been questioned (7,10) because of the experimental conditions employed, and the manipulations performed on these cells.

A convenient approach to studies on the role of hexokinase in HMP seems to be provided by the use of cells with different hexokinase levels.

In the present investigation we show the results obtained by preparing human RBC with increased or reduced hexokinase activity. This has been

ABBREVIATIONS: RBC, Red blood cells; HMP, hexose monophosphate pathway, G6PD glucose 6-phosphate dehydrogenase.

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possible by a procedure of encapsulation of homogeneous hexokinase or purified anti-hexokinase IgG based on hypotonic hemolysis, isotonic resealing and reanneling (11, 12). The main conclusion that can be drawn from the data we have obtained is that a different rate of HMP under oxidative stress is observed changing the hexokinase activity level and that RBC with hexokinase levels comparable to those found in hexokinase-deficiency are not able to respond to oxidizing agents with increased HMP rates.

MATERIALS AND METHODS

Enzymes, coenzymes and substrates were obtained from Sigma Chem. Co. D -[1 ^{14}C] - glucose (58 mCi/mmol) was obtained from the Radiochemical Centre, Amersham U.K., Protein A-Sepharose CL-4B was from Pharmacia, Uppsala, Sweden.

Enzymes were determined by the Beutler methods (13) and glucose utilization in the HMP by measurement of $^{14}CO_2$ production using [1-^{14}C] - glucose as previously described (14) except that all incubations were in autologous plasma instead of buffer, at an hematocrit of 25 %.

Homogeneous human hexokinase type I was prepared from terms placenta as described in ref. 15 with a specific activity of 190 U/mg protein. Antiserum againist homogeneous hexokinase was raised in rabbits immunized with homogeneous hexokinase. The first injection was with complete Freund's adjuvant followed by two further injections of enzyme at 10-day intervals. Each injection consisted of 80-100 μg of protein. IgG were prepared from this serum by chromatography on immobilized Protein A and elution by sodium citrate 0.1M, pH 3.5. 50 μg of IgG were found to be able to inactivate 0.25 units of hexokinase activity.

Encapsulation of hexokinase or anti-hexokinase IgG in human erythrocytes was obtained according to Ropars et al. (12). This procedure involves three sequential steps, i.e. hypotonic hemolysis, isotonic resealing and reannealing of erythrocytes. Briefly, blood was collected in heparin and centrifuged at 2,500 rpm at 4°C to separate the plasma that was maintained at 0°C until use. Erythrocytes were washed twice in 5 mM sodium phosphate buffer, pH 7.4, containing 0.9% (w/v) NaCl and 5 mM glucose and finally resuspended in the same buffer containing hexokinase or anti-hexokinase IgG at a hematocrit of 70% in a dialysis tube. Hypotonic lysis was obtained by dialysis of 2 ml of cell suspension in a Falcon 50 ml sterile tube containing 10 mM sodium phosphate, 10 mM sodium bicarbonate, 20 mM glucose, pH 7.4, and rotated at 15 rpm for 1 h at 4°C. The hemolysate was then collected and 1 vol. of resealing solution (5 mM adenine, 100 mM inosine, 100 mM sodium pyruvate, 100 mM sodium phosphate, 100 mM glucose, 12% (w/v) NaCl, pH 7.4) was added to every 10 vol. of hemolysate. Reanneling of the cells was then obtained by incubation at 37°C for 20 min. Three additional washings of lysed and resealed erythrocytes were performed at 4°C with a physiological saline solution and finally resuspended in their native plasma and utilized for metabolic studies.

RESULTS

HMP in erythrocytes overloaded with hexokinase

Human erythrocytes were overloaded with homogeneous human hexokinase up to 4.77 $\pm$ 0.75 μmoles/min/ml RBC, a value about 15 times higher than that of

corresponding native (untreated cells) or unloaded RBC (erythrocytes processed as for the entrapment technique but without any addition of hexokinase). Estimation of HMP rates in these cells in the presence of different concentrations of methylene blue (0 to 100 μM) provided the results shown in Fig. 1. The native and unloaded erythrocytes showed values almost identical among themselves so that in Fig. 1 they are shown together as "controls". The HMP rates in the hexokinase overloaded RBC are not significantly different from controls in the absence of methylene blue, but showed values 1.5-times higher in the presence of methylene blue. Encapsulation of lower amounts of hexokinase than above resulted in HMP rates, in the presence of methylene blue, that do not increase linearly with the hexokinase level. In fact RBC with hexokinase activity of 1.5 μmoles/min/ml RBC showed an HMP rate in the presence of 100 μM methylene blue that is 31$\pm$ 1.6 nmoles/min/ml RBC. In other words, by increasing the hexokinase activity it is possible to obtain RBC with increased HMP rates under conditions of oxidative stress, although a linear relationship between hexokinase activity and HMP can not be expected because of the complexity of the regulation of hexokinase and HMP in human erythrocytes.

HMP in erythrocytes with encapsulated anti-hexokinase IgG

To obtain RBC with hexokinase activity lower than controls we have prepared antibodies that inactivate the hexokinase activity and loaded them in human RBC as described above for hexokinase. As a result we were able to

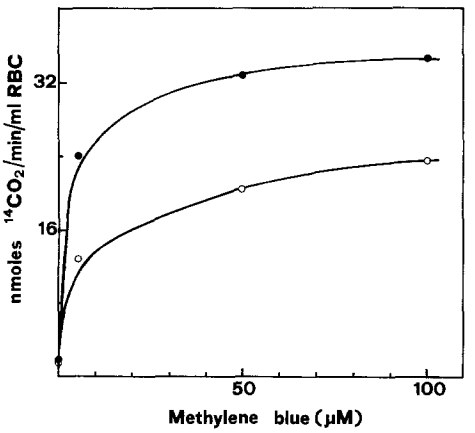

Fig. 1. HMP in human RBC overloaded with hexokinase (●) and normal controls (○), at different concentrations of methylene blue.

prepare RBC with hexokinase activity of 40 ± 2 nmoles/min/ml RBC which represents a value of only 12% of the activity found in the control RBC. The HMP rates in the presence and absence of methylene blue in these cells are shown in Table 1.

DISCUSSION

Many reports in the last years have indicated hexokinase as a control step in the rate of glucose utilization in the HMP of human erythrocytes under conditions of oxidative stress (8-10). However in some papers (7 and references therein) the intracellular distribution of coenzymes has been claimed to be an important mechanism in the control of HMP. The possibility of artefactual observations has been suggested by many Authors (7, 10) to justify the differences among the roles of one or another enzyme in HMP, but although plausible, these explanations have not convincingly provided a definitive answer to what step could limit the ability of human RBC to respond to an oxidative stress. The approach we used of loading RBC with hexokinase to have cells with high content of enzyme, or loading with inactivating antibodies to have cells with reduced enzyme levels, seems useful in understanding at least the role of hexokinase in the control of HMP under conditions of oxidative stress. In fact loading with hexokinase substantially increases the maximal

Table I - HMP in human erythrocytes with different levels of hexokinase activity

Red blood cells	Hexokinase activity (nmoles/min/ml RBC)	Hexose monophosphate shunt (nmoles/min/ml RBC)	
		/	with 100 μM Met Blue
Controls	330 ± 100	1 ± 0.1	24 ± 1.7
Unloaded	320 ± 100	1.3 ± 0.1	25 ± 1.2
Loaded with Anti-hexokinase IgG	40 ± 2	0.9 ± 0.1	3 ± 0.3
Loaded with Hexokinase	4,770 ± 750	1.7 ± 0.2	36 ± 1.8

All values are mean ± S.D. of at least five different experiments.

rate of HMP activity (from 25 ± 1.2 nmoles/min/mol RBC to 36 ± 1.8) while loading with anti-hexokinase IgG dramatically lowers the rate to 3 ± 0.3 nmols/min/ml RBC. The last result, is considerably greater than should be expected from hexokinase activity measured in hemolyzate of the anti-hexokinase loaded erythrocytes (40 ±2 nmoles/min/ml RBC). This presumably occurs because of the large dilution involved in the hexokinase assay (about 400 fold), and the fact that the normal concentration of hexokinase in erythrocytes is ca. 2 x 10^{-8}M, falling in the assay to ca. 10^{-10}M, which could easily be well below the Kd of the hexokinase-anti hexokinase IgG complex (on the basis of typical values around 10^{-7} to 10^{-9}). Hence, real hexokinase activity in the anti-hexokinase loaded erythrocytes could be expected to be much lower than 40, and, considering the regulatory role that many cellular compounds have on the enzyme (16), perhaps it could approach the value of 3 found in the last column of Table I, thus avoiding the problem of having to account for an apparent ten fold drop in activity from in vitro to in vivo. The data obtained at 100 μM methylene blue also show that plotting the HMP rates against hexokinase activity (not shown) produces a biphasic pattern with a fast increase of HMP starting from hexokinase levels of 40 to 330 nmoles/min/ml RBC and a slow increase at higher hexokinase activity. So, although we can certainly conclude that control of HMP under conditions of oxidative stress is exerted by hexokinase, factor(s) other than the enzyme itself become important in the control of HMP rates at higher hexokinase levels. Finally, the low hexokinase levels we have studied are commonly seen in cases of hexokinase deficiency (17) and suggest that the erythrocytes of these patients are not able to adequately increase the HMP rates in the presence of oxidizing agents as commonly found in cases of G6PD deficiency.

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