THE MECHANISM OF RED-CELL HEXOKINASE INHIBITION INDUCED BY OXIDATION OF INTRACELLULAR GLUTATHIONE AND ITS RELATION TO DRUG SENSITIVITY

J. Mager, A. Razin, A. Hershko and G. Izak

Department of Biochemistry, Hebrew University-Hadassah Medical School and School of Dentistry and Department of Medicine, Hadassah University Hospital, Jerusalem, Israel.

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Washed human red blood cells (RBC) incubated in phosphate-buffered saline supplemented with 3×10^{-2}M acetylphenylhydrazine (APH), exhibited a rapid loss of reduced glutathione (GSH), followed by a steep fall of their ATP content. Addition of glucose at the start of incubation with APH, markedly reduced the extent of these alterations in normal RBC, but afforded little or no protection against the noxious effects of APH on glucose-6-phosphate (G6PD)-deficient erythrocytes (Table I). The

Table I

Decline of GSH and ATP in normal and G6PD* -deficient erythrocytes incubated with APH**; effects of glucose and inosine

Substances added to the erythrocyte suspension	Normal erythrocytes		G6PD*-deficient erythrocytes	
	GSH	ATP	GSH	ATP
	μmoles per ml erythrocytes		μmoles per ml erythrocytes	
None	1.1	0.70	1.1	0.64
APH**, 3×10^{-2}M	0.0	0.32	0.0	0.24
Glucose, 10^{-2}M	1.8	0.99	1.4	0.96
Glucose, 10^{-2}M plus APH**, 3×10^{-2}M	0.8	0.75	0.0	0.43
Inosine, 10^{-2}M	1.8	0.96	1.4	1.00
Inosine, 10^{-2}M plus APH**, 3×10^{-2}M	0.6	0.86	0.0	0.89

The erythrocyte suspensions in phosphate-saline (pH 7.4) were incubated in the presence of various supplements, as indicated, at 37°C with continuous shaking for 2 hours. GSH was determined according to Beutler et al. (1963) and ATP was estimated by the luciferin-luciferase method, as previously described (Razin and Mager, 1964).

*Glucose-6-phosphate dehydrogenase; **acetylphenylhydrazine

observed responses of the red-cell GSH to the various conditions of treatment with APH were in accord with the original findings of Beutler et al. (1957). On the other hand, the ATP decreases occurring under our experimental conditions (see legends to Table 1), were much more pronounced than the essentially similar changes described by Mohler and Williams (1961) in whole blood exposed to phenylhydrazine.

Subsequent experiments revealed that the protective effect of glucose on normal erythrocytes was no longer elicitable, if its addition was postponed until the GSH content of the cells became exhausted in the course of incubation with APH. The decline of ATP proceeded unabated, even if following preincubation with APH, the drug was subsequently removed by thorough washing of the cells (Table II).

Table II

Deoxyglucose-induced depletion of red-cell ATP and its prevention by pretreatment with APH or cystamine; antagonistic effect of NEM*

Substances added to the erythrocye suspension at preincubation	ATP μmoles per ml erythrocytes			
	start of reincubation	end of reincubation		
		no addition	glucose 10^{-2}M	2-deoxy-D-glucose 10^{-2}M
None (control)	0.65	0.55	0.78	0.14
APH, 3×10^{-2}M	0.53	0.35	0.35	0.32
Cystamine, 7.5×10^{-3}M	0.41	0.32	0.33	0.31
NEM*, 3×10^{-3}M	0.55	0.35	0.23	0.17
NEM**, 3×10^{-3}M followed by APH, 3×10^{-2}M	0.35	0.21	0.13	0.11
NEM**, 3×10^{-3}M followed by cystamine, 7.5×10^{-3}M	0.35	0.17	0.12	0.09

Following preincubation for 2 hours in the presence of the various substances, as specified, the erythrocytes were washed twice and resuspended in phosphate-saline (pH 7.4) and then reincubated for another 2 hours. The GSH content at the start of reincubation was 1.8 μmoles per ml erythrocytes in the control and nil in all other instances. Other details were as in Table 1.

*N-ethylmaleimide; **N-ethylmaleimide added 10 minutes prior to APH

It appeared likely that the progressive decay of ATP consequent upon the treatment of RBC with APH, reflected some permanent injury of their glycolytic system, since the latter pathway is known to constitute the only device of these cells for ATP replenishment (see Bartlett and Shafer, 1961). This assumption was borne out by the nearly complete arrest of glucose consumption observed in erythrocytes, which have been pretreated with APH, and then washed and resuspended in fresh medium without APH added (see also Lõhr and Waller (1961) and Kosower et al. (1963).

Further insight into the nature of the APH-induced metabolic disturbance was provided by the observation that inosine, when added simultaneously with APH, obviated the decline of ATP in both normal and G6PD-deficient erythrocytes, notwithstanding its failure to prevent the destruction of GSH (Table I). However, once the ATP content of the RBC has been substantially reduced by suitable pretreatment with APH, restoration of the normal level of ATP could be achieved by reincubation with adenosine or a mixture of adenine and inosine but not by supplementation with inosine alone. It appeared reasonable to attribute the protective influence of inosine to the ability of this nucleoside to bypass the hexokinase step, as a result of its phosphorolytic cleavage to hypoxanthine and ribose-1-phosphate (cf. Bartlett and Shafer, 1961). On the other hand, the essentiality of adenine or adenosine for the reversal of the ATP depletion was in line with the additional observation that the break-down of ATP in APH-treated RBC was attended by a considerable deamination of its adenine moiety to hypoxanthine (Hershko et al.), which apparently cannot be efficiently utilized by human erythrocytes for the resynthesis of adenine nucleotides (cf. Bartlett and Shafer, 1961).

Thus, the above results pointed to the hexokinase reaction as the likely site of the major biochemical lesion. This conclusion was corroborated by the finding that pretreatment of RBC with APH prevented the depletion of their ATP pool occurring on incubation in the presence of 2-deoxy-D-glucose (Table II). This glucose analogue consumes ATP in a wasteful process of "dead-end" phosphorylation mediated by

hexokinase. The accumulating reaction product (2-deoxyglucose-6-phosphate), being incapable of undergoing further metabolism (cf. Ibsen et al., 1958), is unable to requite the mounting "ATP debt".

The failure of glucose to protect G6PD-deficient erythrocytes against the deleterious effect of APH on their hexokinase activity, appeared to be related to the intrinsic "GSH instability" of these cells (Beutler et al., 1957). This interpretation, called to mind the description by Eldjarn and Bremer (1962) of a similar disturbance, produced by cystamine and a variety of other disulfide compounds capable of permeating the red blood cell. The significance of this analogy was further enhanced by the recognition of the role of GSSG formation as an obligatory step in the mechanism underlying the blockade of hexokinase. Experiments designed to examine the effect of GSH sequestration with the aid of N-ethylmaleimide (NEM) (see Jacob and Jandl, 1962) revealed that erythrocytes treated with NEM at a dose sufficient to bind all the GSH present in the cells, showed no derangement of hexokinase activity, when examined by the above outlined 2-deoxyglucose test. Moreover, addition of NEM prior to the start of incubation with APH, eliminated subsequent occurrence of hexokinase inhibition, presumably by rendering the GSH unavailable to oxidation to GSSG (Table II).

However, despite all the evidence presented above, hemolyzates derived from erythrocytes treated with APH under the standard conditions employed by us, showed no impairment of hexokinase activity, as determined in a system coupled to the NADP-linked G6PD. The latter results were consistent with the interpretation that the APH-induced oxidation of the intracellular GSH was not associated with an irreversible inactivation of hexokinase but entailed rather the accumulation of a substance which specifically interfered with the action of this enzyme. Furthermore, it seemed reasonable to ascribe the failure of the enzymic test to detect the presence of the hexokinase inhibitor to its excessive dilution, inherent in the necessity of employing minute amounts of hemolyzate in conformity with the requirements of the spectrophotometric assay.

The non-identity of the hypothetic inhibitor with GSSG was inferred from the fact that the extent of reduction of GSSG to GSH in a coupled multi-system reaction comprising hexokinase, glucose-6-phosphate dehydrogenase and GSSG reductase, showed stoichiometric agreement with the rate of NADPH formation observed in the absence of GSSG. The appearance of an unidentified hexokinase inhibitor in erythrocytes preincubated with APH or cystamine could be finally demonstrated by using sufficiently concentrated hemolyzates (equivalent to 1 vol. of RBC per 3 vol. of final reaction mixture) and determining the reduction of GSSG as a function of the hexokinase-dependent phosphorylation of glucose (Table III). Furthermore, incubation of stroma-free hemolyzates with added GSSG (3×10^{-3}M) was found to result in a rapidly advancing inhibition of the endogenous hexokinase activity, which could be fully reversed, however, by appropriate dilution. Work aimed at the elucidation of the nature of the inhibitory substance is being continued.

Table III

Inhibition of hexokinase-dependent reduction of GSSG to GSH in hemolyzates derived from erythrocytes pretreated with APH or cystamine; protective effect of glucose

Substances added to the erythrocyte suspension at preincubation	GSH produced μmoles per hour per ml erythrocytes
None	13.0
Glucose, 10^{-2}M	14.5
APH, 3×10^{-2}M	1.5
Glucose, 10^{-2}M plus APH 3×10^{-2}M	14.5
Cystamine, 7.5×10^{-2}M	1.5
Glucose, 10^{-2}M plus cystamine, 7.5×10^{-3}M	14.5

Suspensions of normal erythrocytes in phosphate-saline (pH 7.4) were incubated for 2 hours in the presence of various substances, as detailed in the Table. The cells were then washed twice with phosphate-saline and hemolyzed by diluting with an equal volume of distilled water. The hemolyzates were freed of the stromata by centrifugation for 15 minutes at 21,000 x g and the supernatants were used as source of enzymes. The assays were performed at 37°C, using the following reaction mixture: 1 ml hemolyzate, 5×10^{-2}M Tris buffer (pH 7.4), 4×10^{-3}M glucose, 3×10^{-3}M GSSG, 10^{-3}M ATP, 3×10^{-3}M creatine phosphate, 20 μg creatine kinase 5×10^{-3}M and 10^{-3}M NADP; total volume: 1.5 ml. Other details as in Table I.

While this study was in progress, Kosower et al. (1964) reported preliminary observations on the partial destruction of hexokinase in G6PD-deficient erythrocytes, following their exposure to APH for periods which were about 3 to 4 times longer than those employed in our experiments. The relevance of these data to the mechanism of glycolysis inhibition and ATP decay is still open to critical evaluation.

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