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THE METABOLIC EFFECTS OF OXALATE ON INTACT RED BLOOD CELLS *

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Summary

The metabolic effects of oxalate on pyruvate kinase were studied in intact human red blood cells and compared to the spontaneous modifications induced by congenital pyruvate kinase deficiency. In normal cells, oxalate ($2—3 \cdot 10^{-4}$ M) produces a large increase of the monophosphoglycerates, phospho*enol*-pyruvate pool and decrease of pyruvate concentration as a result of pyruvate kinase inhibition; it does not significantly modify 2,3-diphosphoglycerate level, ATP formation or overall glycolytic activity. Those effects of oxalate are not due to Mg^{2+} chelation. A similar metabolite pattern is observed in vivo in erythrocytes with congenital pyruvate kinase deficiency, in which ATP concentration and glycolytic activity are described. These cells are more sensitive to oxalate than normal ones.

Results are discussed with reference to the rate-limiting character of normal or congenitally deficient pyruvate kinase.

Introduction

We have recently shown [1] that oxalate exerts a dual kinetic effect on L-type pyruvate kinases (EC 2.7.1.40) from liver and erythrocytes. When allosteric pyruvate kinase is under T state (at low phospho*enol*pyruvate concentrations or in the absence of fructose 1,6-diphosphate) oxalate behaves as a

* Preliminary report of this study has been presented to the FEBS special meeting on Enzyme, April 1979, Dubrovnik [13].

slight allosteric activator; on the contrary, in the presence of micromolar concentration of fructose 1,6-diphosphate and/or at higher phospho*enol*pyruvate concentrations, the protein is shifted to R state and oxalate becomes a powerful competitive inhibitor with respect to phospho*enol*pyruvate (K_i = $8 \cdot 10^{-6}$ M). Indeed, Reed and Morgan [2] had already described such an inhibition on muscle pyruvate kinase and shown that it was due to structural analogy between oxalate and phospho*enol*pyruvate.

Oxalate is normally present in blood at a concentration of about $2—3 \cdot 10^{-5}$ M [3] and enters freely into human red cells [4]. Thus oxalate might be considered as one of the most powerful physiological inhibitor of pyruvate kinase. In the present report the modifications of steady-state concentrations of glycolytic intermediates produced by low concentrations of oxalate are studied. They are compared to the metabolic changes found in hereditary defects of erythrocyte pyruvate kinase.

Materials and Methods

All substrates, coenzymes and auxiliary enzymes for spectrophotometric determination of glycolytic intermediates were purchased from Boehringer (Mannheim).

Determination of glycolytic intermediates in red blood cells. Estimation of glycolytic metabolites in red cells from patients with hereditary pyruvate kinase defect was carried out on whole blood as already described [5] and compared with those from healthy subjects. Estimation was performed with an Eppendorf photometer at 366 or 334 nm according to reported methods [5]. 2,3-Diphosphoglycerate was assayed by the phosphoglycolate method described by Rose and Liebowitz [6].

Study of the effect of oxalate on steady-state level of metabolites. Heparinized blood (freshly drawn from healthy subjects) was centrifuged to eliminate leucocytes and platelets. Red blood cells were washed twice with 2 vols. of isotonic KCl, then suspended in 2 vols. of the following medium (pH 7.4): 115 mM NaCl, 4 mM KCl, 5 mM $MgCl_2$, 16 mM NaH_2PO_4, 3.5 mM glucose. The cell suspension (4.2 ml) was distributed into 25-ml rubber-stoppered Erlenmeyer flasks and preincubated for 2 h at 37° C with a shaking rate of 100 cycles/min, after which three different series of samples were prepared: (i) immediately deproteinized (controls A); (ii) reincubated at 37° C after addition of 100 µl of the above buffer without oxalate (controls B), and (iii) reincubated as (ii) after addition of 100 µl of oxalate-containing buffer. The times and the final oxalate concentrations are indicated in the legends of the tables and figures. The contents of the flasks were deproteinized by addition of 3 ml of ice-cold 2 M perchloric acid. After 10 min at 4° C the acid extracts were centrifuged ($5000 \times g$, 10 min) and neutralized with solid $KHCO_3$.

Pyruvate was immediately assayed, followed by the determination of fructose 1,6-diphosphate, triose phosphates and phospho*enol*pyruvate; other metabolites could be assayed on the following days in neutralized extracts stored at −15° C.

Results

Comparison of metabolite steady-state levels measured in vivo and in incubated red cells

Table I compares the concentration of metabolites in: (i) immediately deproteinized blood; (ii) washed red cells, and (iii) incubated red cells. In washed red cells the redox state calculated as the ratio lactate/pyruvate is strongly modified, therefore increases the level of fructose 1,6-diphosphate and triose phosphates [7]. However, after 2 h of incubation the physiological steady-state level of glycolytic intermediates was nearly restored and fructose 1,6-diphosphate reached a value close to the one found in whole blood. It is important to point out that the values for these metabolic intermediates remained unchanged for another period of 2 h of incubation after which several of these compounds diminished quite significantly.

Metabolic changes induced by oxalate

The variations in the steady-state level of glycolytic substrates and adenine nucleotides following addition of oxalate have been studied at different concentrations of oxalate (Table II) and at different incubation times (Fig. 1).

The smallest oxalate concentration leading to significant metabolic changes was $2 \cdot 10^{-4}$ M. i.e. seven times the normal physiological range. Lower concentrations did not produce any change even though the incubation time was extended to 120 min. Beyond this time metabolic disturbances appeared in the controls. At $2 \cdot 10^{-4}$ M oxalate, there was a significant raise of phospho*enol*-pyruvate and monophosphoglycerates concentrations (Table II). Pyruvate con-

TABLE I

METABOLITE CONCENTRATIONS IN RED BLOOD CELLS UNDER DIFFERENT EXPERIMENTAL CONDITIONS

Concentrations are expressed as nanomoles per ml of packed erythrocytes except for lactate and pyruvate which are expressed as nmol/ml of whole blood or whole cell suspension. Values for whole blood are means ± SD from 16 determinations. Values for incubated red cells are means ± SD from 21 determinations (controls A and B) for phosphorylated metabolites which do not vary significantly during the incubations, and from 13 determinations (control A only) for pyruvate and lactate whose concentrations are time-related. Controls A and B are defined in Materials and Methods.

	Whole blood	Washed red blood cells	Incubated red cells
Glucose 6-phosphate	29.1 ± 6.8	23	72.2 ± 8.3
Fructose 6-phosphate	10.2 ± 1.8	7	25.4 ± 3.2
Fructose 1,6-diphosphate	1.2 ± 0.4	48	4.7 ± 1.1
Triose phosphatase	15.2 ± 4.9	126	16.5 ± 4.4
3-Phosphoglycerate	66.3 ± 7.4	70	73.1 ± 8.9
2-Phosphoglycerate	11.8 ± 2.5	8	9.0 ± 2.5
Phospho*enol*pyruvate	17.4 ± 3.8	16	21.1 ± 4.4
ATP	1527 ± 180	1320	1592 ± 177
ADP	157 ± 21	240	123 ± 16
AMP	26 ± 10	37	26 ± 8
2,3-Diphosphoglycerate	4780 ± 610	5680	5038 ± 476
Pyruvate	82 ± 30	2	61 ± 15
Lactate	1400 ± 416	160	1702 ± 158

TABLE II

EFFECTS OF VARIOUS OXALATE CONCENTRATIONS AND PYRUVATE ADDITION ON GLYCOLYTIC INTERMEDIATES IN RED CELLS INCUBATED FOR 20 MINUTES

Metabolic concentrations are expressed as nmol/ml packed cells, except for pyruvate and lactate (in nmol/ml of cell suspension). Glucose consumption and lactate production are expressed as μmol/ml packed cells per h. The control values for phosphorylated intermediates are the means of controls A and B (Table I). For glucose, pyruvate and lactate it was not possible to use the means of controls B: (i) there are important fluctuations in the overall rate of glycolysis (S.D. is about 20% of the mean); (ii) incubation times were not the same in all experiments (see for example Fig. 1). Thus the controls used in this table are the mean values obtained with the same cells simultaneously incubated.

	Controls	$2 \cdot 10^{-4}$ M oxalate No pyruvate added (mean of two experiments)	$3 \cdot 10^{-4}$ M oxalate		$5 \cdot 10^{-4}$ M oxalate	
			No pyruvate added (mean of two experiments)	$3 \cdot 10^{-4}$ M pyruvate (one experiment)	No pyruvate added (mean of two experiments)	$3 \cdot 10^{-4}$ M pyruvate (one experiment)
Glucose 6-phosphate	72.2 ± 8.3	68	69	64	55	64
Fructose 6-phosphate	25.4 ± 3.2	24	21	23	17	23
Fructose 1,6-diphosphate	4.7 ± 1.1	4.4	3.8	3.7	8.6	4.0
Triose phosphates	16.5 ± 4.4	15.3	12.9	11.3	40	11.3
3-Phosphoglycerate	73.1 ± 8.9	99	117	110	160	159
2-Phosphoglycerate	9.0 ± 2.5	12	19.5	17	29	29
Phospho*enol*pyruvate	21.1 ± 4.4	28	41	39	58	62
ATP	1592 ± 177	1507	1413	1401	1414	1401
ADP	123 ± 16	117	119	132	149	137
AMP	26 ± 8	18	24	18	34	28
2,3-Diphosphoglycerate	5038 ± 476	4625	4471	4681	4745	4954
Pyruvate	85	79	49	217	38	260
Lactate	1740	1717	1660	1685	1712	1643
Glucose consumption	1.09	1.20	1.11	1.30	1.14	—
Lactate production	2.69	2.85	2.56	2.40	2.65	2.51

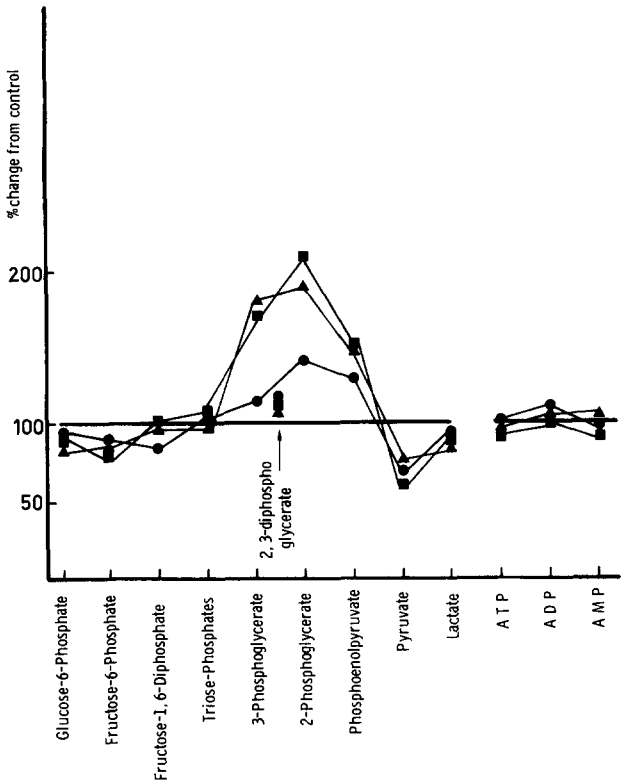

Fig. 1. Effect of incubation time on metabolic modifications induced by $3 \cdot 10^{-4}$ M oxalate. The 100% level is represented by mean values obtained in incubated red cells (Table I, 3rd column), except for pyruvate and lactate where the reference is a simultaneously incubated control. Incubation time: ●, 0.5 min; ■, 2 min; ▲, 5 min.

centration decreased and this metabolic cross-over between pyruvate and phospho*enol*pyruvate is consistent with an inhibition of pyruvate kinase. There was no modification in nucleotide level.

At $3 \cdot 10^{-4}$ M the metabolic cross-over was greatly enhanced without any detectable secondary repercussion.

At $5 \cdot 10^{-4}$ M a significant increase of fructose 1,6-diphosphate and triose phosphate was also observed and a secondary cross-over point appeared at the phosphofructokinase step. These secondary changes were completely abolished if $3 \cdot 10^{-4}$ M pyruvate was added to the incubation medium simultaneously with oxalate (Table II). The lactate/pyruvate ratio was then artificially brought to 6—8, being 20 in the control.

In Fig. 1 the influence of varying incubation times on the metabolite pattern of glycolytic intermediates is shown at $3 \cdot 10^{-4}$ M oxalate. The metabolite cross-over at pyruvate kinase levels is immediately observed (0.5 min) and is maximal after 2 min. No other significant changes were observed, ATP was very stable. In all these incubations no modification of overall glycolysis (assayed as glucose consumption or lactate production) was noticed.

TABLE III

METABOLIC PATTERN IN HEREDITARY PYRUVATE KINASE DEFECT WITHOUT RETICULOCYTOSIS

Metabolites were determined in whole blood as described in Material and Methods. Their concentrations are expressed as nmol/ml packed cells. Glucose consumption of incubated cells was: in controls (30 determinations) 1.31 ± 0.24 μmol/ml packed cells per h; in patient A, 0.73; in patient B, 1.39.

	Controls (n = 16)	Patient A (mean of two determinations)	Patient B
Glucose 6-phosphate	29.1 ± 6.8	19.3	25.4
Fructose 6-phosphate	10.2 ± 1.8	7.0	8.5
Fructose 1,6-diphosphate	1.2 ± 0.4	1.1	0.7
Triose phosphates	15.2 ± 4.9	9.1	11.8
3-Phosphoglycerate	66.3 ± 7.4	107	125
2-Phosphoglycerate	11.8 ± 2.5	23	24
Phospho*enol*pyruvate	17.4 ± 3.8	40	34
ATP	1527 ± 180	517	710
ADP	157 ± 21	124	157
AMP	26 ± 10	38	54
2,3-Diphosphoglycerate	4780 ± 610	5090	5301

Comparison of the oxalate-induced changes at pyruvate kinase level with metabolic disturbances found in hereditary pyruvate kinase deficiency

Table III shows the metabolic pattern of two patients (one of them was studied twice [8]) whose reticulocytosis did not exceed 1%. The predominant

TABLE IV

EFFECT OF OXALATE ON GLYCOLYTIC METABOLITES OF INCUBATED RED CELLS FROM A PATIENT WITH HEREDITARY PYRUVATE KINASE DEFECT WITH 20% RETICULOCYTOSIS

Metabolite concentrations are expressed as nmol/ml packed cells except for pyruvate and lactate (in nmol/ml of cell suspension). Glucose consumption and lactate production are expressed as μmol/ml packed cells per h. n.m., not measurable.

	Deficient red cells incubated for 20 min		
	Without oxalate	With $3 \cdot 10^{-4}$ M oxalate	With $5 \cdot 10^{-4}$ M oxalate
Glucose 6-phosphate	179	82	61
Fructose 6-phosphate	65	24	28
Fructose 1,6-diphosphate	16	183	237
Triose phosphates	90	505	534
3-Phosphoglycerate	314	638	637
2-Phosphoglycerate	32	65	65
Phospho*enol*pyruvate	104	255	261
ATP	1 408	1 125	1 020
ADP	92	255	316
AMP	36	82	117
2,3-Diphosphoglycerate	10 676	10 774	10 774
Pyruvate	51	4	n.m.
Lactate	1 131	1 195	1 109
Glucose consumption	1.60	1.72	1.72
Lactate production	1.86	1.96	1.83

variation was a high level of phospho*enol*pyruvate and monophosphoglycerates as observed after incubation of normal red blood cells in $3 \cdot 10^{-4}$ M oxalate (Table II; Fig. 1). 2,3-Diphosphoglycerate was slightly elevated and its variation was small when compared to phospho*enol*pyruvate. Contrary to the experiments mentioned above, in these two patients ATP was obviously low and for one of them glycolysis was greatly diminished, two facts which are not observed in oxalate incubations (Table II).

Effects of oxalate on red cell metabolism in a patient with hereditary pyruvate kinase deficiency

Table IV shows the metabolic changes induced by oxalate at 3 and $5 \cdot 10^{-4}$ M, similar to the concentrations used in normal red blood cells (Table II). The level of metabolites greatly differs from the normal control owing to high reticulocytosis (20%) and the pyruvate kinase defect. It clearly appears that $3 \cdot 10^{-4}$ M oxalate, which on normal red blood cells only increases the phospho*enol*pyruvate and monophosphoglycerates concentration without secondary metabolic cross-over, strongly modifies the first steps of glycolytic pathway and decreases significantly ATP level in pyruvate kinase-deficient cells. However, it has no significant effect on overall glycolytic activity.

Discussion

Some of the oxalate-induced metabolic modifications might be due to the formation and precipitation of an oxalate-Mg^{2+} complex. The solubility product of this complex is $8.6 \cdot 10^{-5}$, and, at the highest oxalate concentration used, the product [oxalate] $\times$ [Mg^{2+}] was only $2.5 \cdot 10^{-6}$. Moreover, $3 \cdot 10^{-4}$ M citrate, a more potent Mg^{2+} chelator than oxalate, and which can cross red blood cell membranes almost as freely as oxalate, had no effect on pyruvate kinase activity inside cells. The simultaneous addition of citrate + oxalate elicited only the effects of oxalate (results not shown).

In incubated cells (Table I), glucose, lactate, pyruvate, phosphorylated intermediates and nucleotides were near the physiological concentrations. In the absence of added oxalate, the level of all metabolites remained constant for at least 120 min. Glucose consumption (1.20—1.60 μmol/h per ml of cells) was linear with time and strongly correlated to lactate production. In these conditions, the immediate modifications induced by oxalate (Table II) should proceed from the already reported [1] kinetic inhibition of pyruvate kinase.

However, if we compare the results with kinetic data, we cannot ascertain the allosteric activation of pyruvate kinase since fructose 1,6-diphosphate is always present at micromolar concentration and pyruvate kinase is likely predominantly under R state. In this condition, the only effect is the inhibition of pyruvate kinase by oxalate in the 10^{-4} M range.

The oxalate concentration ($3 \cdot 10^{-4}$ M) able to raise 2-fold phospho*enol*pyruvate level and lower pyruvate to the same extent is about 40-fold higher than the kinetically measured K_i ($8 \cdot 10^{-6}$ M) [1]. This may be due to the presence of various effectors of pyruvate kinase (e.g., fructose 1,6-diphosphate, glucose 1,6-diphosphate, 2,3-diphosphoglycerate and ATP) in intact cells.

We presume that oxalate primarily reacts at the level of pyruvate kinase. At

$5 \cdot 10^{-4}$ M oxalate, other metabolic variations like increased levels of triose phosphate and fructose 1,6-diphosphate may simply result from mass action ratio of the glyceraldehyde phosphate dehydrogenase-phosphoglycerate kinase system. The increased lactate/pyruvate ratio reflects a decreased NAD^+/NADH ratio which tends to elevate triose phosphate concentration followed by fructose 1,6-diphosphate which activates phosphofructokinase [9] and then leads to decreased hexose monophosphates. When pyruvate is added (Table II), the NAD^+/NADH ratio increases and as a consequence triose phosphate and fructose 1,6-diphosphate fall to normal values. Phosphofructokinase is no longer activated and normal hexose monophosphate concentration is recovered.

If we compare the effects of oxalate (Table III) to the metabolic pattern found in congenital pyruvate kinase deficiency, a similar cross-over at the level of pyruvate kinase is observed but three important differences appear:

1. In congenitally deficient cells, overall glycolytic activity and ATP level decrease, which is not the case in normal cells at the oxalate concentrations used by us. It may be postulated that in the latter case the inhibition of pyruvate kinase is overcome by increased phospho*enol*pyruvate concentration: pyruvate kinse is presumably not saturated by its substrate under physiological conditions [10], and oxalate is a competitive inhibitor towards phospho*enol*-pyruvate. On the contrary, in genetic defects, the residual activity of pyruvate kinase is too low to be normalized by an increased substrate concentration; as a consequence glycolytic activity and ATP generation are impaired.

2. The secondary metabolic cross-over at the phosphofructokinase step does not appear in freshly deproteinized, but not reincubated deficient cells (Table III). In circulating blood there is a continuous supply of pyruvate which allows reoxidation of NADH as observed when pyruvate is added together with oxalate (Table II).

3. It has been reported that congenitally pyruvate kinase-deficient cells have strong increases in 2,3-diphosphoglycerate [11] which were not confirmed in the cases reported here (patients with low reticulocyte count), where it is not much elevated and always remains far below the relative increase of phospho*enol*-pyruvate and monophosphoglycerates. Indeed a high reticulocyte count is often present in blood from pyruvate kinase-deficient patients. In metabolic studies on six patients with high reticulocytosis we have found [8] that when 2,3-diphosphoglycerate level increases 2-fold, phospho*enol*pyruvate and monophosphoglycerates concentration increases 4—6-fold. Moreover it may be postulated that the large pool of 2,3-diphosphoglycerate slowly reacts to metabolic events able to modify rapidly other metabolic concentrations. However, concentrations of 2,3-diphosphoglycerate and ATP were not appreciably modified by longer incubations (up to 2 h) of normal cells in the presence of oxalate (results not shown).

$3 \cdot 10^{-4}$ M oxalate does not modify the ATP content of normal erythrocytes whereas it impairs ATP formation in reincubated deficient red cells (Table IV). Some commonly used drugs, such as ascorbate, are metabolized to oxalate [12]. Their use in pyruvate kinase-deficient patients might be questioned.

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