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INFLUENCE OF GLUCOSE AND INHIBITORS OF GLYCOLYSIS ON RELEASE OF TOTAL PROTEINS AND ENZYMES FROM HUMAN LEUKOCYTES

M. LAHRICHI, PIERRETTE TARALLO, Y. HOUPERT and G. SIEST

Laboratoire de Biochimie Pharmacologique, Faculté des Sciences Pharmaceutiques et Biologiques, 7, rue Albert Lebrun, 54000 Nancy (France) and Laboratoire du Centre de Médecine Préventive, 2, avenue du Doyen Jacques Parisot, 54500 Vandoeuvre les Nancy (France)

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Summary

We studied the influence of substrates and glycolysis inhibitors on the release of proteins and enzymes from human leukocytes. We show the relationship between metabolic level and percentage of enzyme release.

Leukocyte kept alive in a nutrient-free bicarbonate medium liberate enzymes even though their metabolic state is satisfactory. The addition of glucose to the medium significantly decreases this phenomenon without affecting the energy level. Addition of glycolysis inhibitors increases it, reduces the energy level, and interferes with other metabolic pathways.

The results are discussed and compared to those obtained by other authors using various cell models.

Introduction

Numerous studies have shown that incubated organs or cells such as the heart [1], mouse liver [2], rat diaphragm or muscle [3,4], and ascitic cells [5], released proteins and enzymes into the ambient medium.

In a previous study, we showed that human granulocytes kept alive in a nutrient-free bicarbonate medium also released proteins, even when their cellular metabolism was satisfactory [6]. During the first hour of incubation, such release of proteins was due neither to thermal shock, nor to microtrauma during isolation, but rather to impairment of the membrane. In order to main-

ADP, adenosine diphosphate; AP, alkaline phosphatase (EC 3.1.3.1); ATP, adenosine triphophate; DHAP, dihydroxyacetone phosphate; G3P, glycerol 3-phosphate; β-glu, β-glucuronidase (EC 3.2.1.31); Lac, lactate; LDH, lactate dehydrogenase (EC 1.1.1.27); PK, pyruvate kinase.

tain an energy level capable to sustaining exchange reactions and consequently a certain structural integrity, the cell breaks down its own components, particularly glycogen [7].

On the other hand, addition of glucose to the medium decreases the release of proteins and enzymes, whatever the cell or organ studied [1—4].

However, few studies have sought to establish a relationship between protein and enzyme release and the metabolic state of the cells. Also, human leukocytes have been used very little to this end. Englehardt's work merely touched upon the problem [8].

In this study, therefore, we examine the relationship between metabolic activity in leukocytes and the release of cytoplasmic and granular enzymes.

Materials and methods

1. Isolation of leukocytes

Granulocytes were isolated from venous blood collected under lithium heparinate from healthy, fasting subjects aged 20—40 years. The isolation technique and methods of studying cell purity and viability have already been described [9]. The cellular suspension obtained contained 85—95% polymorphonuclear leukocytes. Granulocytes were contaminated with platelets in a 1 : 1 ratio, but no erythrocytes were visible under the microscope.

2. Composition of incubation media

The media were derived from Krebs-Ringer bicarbonate solution medium and modified according to Esmann [10], to produce the following composition per liter:

100.15 mmol NaCl; 4.7 mmol KCl; 2.5 mmol $CaCl_2$; 1.2 mmol KH_2PO_4; 1.2 mmol $MgSO_4 \cdot 7H_2O$; 15 mmol $NaHCO_3$; 24.4 mmol CH_3COONA; PVP 1% (w/v).

Medium 1 was without glucose, while medium 2 contained 16.5 mmol/l

In both cases, osmolarity was maintained at 309.5 milliosmol/l by modifying the NaCl concentration.

Media were prepared daily and the pH was adjusted to 7.4 by bubbling for 15 min with mixture of CO_2/O_2 (5 : 95, v/v) saturated in water vapor.

All incubation were for about 60 min; by then the rate of protein release was stabilized [6].

3. Measurement of metabolites and of glucose consumption

Levels of the metabolites ATP, ADP, lactate, pyruvate, DHAP and G3P were measured using the methods of Cartier et al. [11], and the NAD^+/NADH ratio was calculated as follows:

$$\frac{NAD^+}{NADH} = \frac{1}{K'} \times \frac{\text{dihydroxyacetone phosphate}}{\text{glycerol 3-phosphate}}$$

$K' = 8.9 \times 10^{-12}$ at pH 7 [12].

* PVP, polyvinylpyrrolidone, PM = 25000-30000 Merck.

The amount of glucose consumed by the cells was determined by the hexokinase method using Boehringer reagent kits.

4. Measurement of proteins and enzymes

After incubation, the cells were separated from the medium by centrifugation at 3000 rpm for 10 min at +4°C. The proteins and enzymes in both the supernatant and the pellet were measured. Proteins were determined using the method of Lowry et al. [13]. The activities of PK, LDH, and alkaline phosphatase were measured using Boehringer reagent kits, and that of β-glucuronidase, by the method of Richterich and Damvalder [14].

Proteins and enzymes were extracted by grinding the cells with an Ultra Turrax at 0°C in the presence of 0.025% Triton X-100, a concentration often used for leukocytes [15,16]. At this Triton concentration PK was inactivated within 30 min. This has already been observed with platelet PK [17].

Percentages of protein and enzyme release were calculated according to the formula below:

$$\frac{\text{Activity in supernatant}}{\text{Activity in pellet + Activity in supernatant}} \times 100$$

All assays were performed in duplicate. Percentages of protein and enzyme released in the various media were compared using the F test and Student's t-test.

Results and discussion

1. Metabolic behavior

1.1 Effect of glucose

In medium 1 (Table I), the lactate produced can only come from cellular glycogen; 80% of the glycogen disappears after one hour of incubation [7]. In medium 2, the lactate comes from consumed glucose, 95% of which is catab-

TABLE I

INFLUENCE OF GLUCOSE ON GLYCOLYSIS IN HUMAN LEUKOCYTES IN VITRO

80×10^6 to 100×10^6 cells were suspended in 1 ml medium and incubated at 37°C for 60 min. The glycolysis metabolites were extracted and measured as described in Materials and Methods. The results are given in $\mu mol/10^{10}$ cells and represent the mean ± standard deviation of 4 assays done in duplicate.

Compounds	Medium 1	Medium 2 (with glucose)
Glucose uptake		508 ± 38
Lactate	640 ± 77	685 ± 126
Pyruvate	12 ± 2.7	12.8 ± 1.4
Dihydroxyacetone phosphate	1.8 ± 0.35	2 ± 0.3
Glycerol 3-phosphate	1.13 ± 0.2	0.74 ± 0.14
ATP	9.8 ± 1.1	10.9 ± 1.2
ADP	2.1 ± 0.2	2.2 ± 0.19
$NAD^+/NADH$	17 600	22 400

olized by the Embden-Meyerhof pathway. Even in the presence of glucose there is some slight glycogenolysis [7].

The ratio of lactate produced to glucose consumed is 1.3, while the theoretical ratio is 2. The theoretical and the experimentally derived ratios differ because some of the lactate produced seems to be incorporated into glycerolipids [18].

The ATP and pyruvate levels did not differ significantly in the two media, and the ATP/ADP ratio of 4.90 indicates a good energy level [19]. These values are close to those found by other authors [7,10,20].

The NAD^+/NADH ratio was 17 600 in the glucose-free medium and 22 400 in the medium containing glucose. Because lactate is an end-reaction product, we preferred the DHAP/G3P ratio to the pyruvate/lactate ratio as an indicator of metabolic state. The DHAP/G3P ratio that we found under various metabolic conditions, even if it does not have a specific value, reflects a near-equilibrium state of G3P dehydrogenase. The value obtained was similar to that published by Marchand et al. [7] but was clearly higher than that calculated by Mintz and Robin [21] using the lactate/pyruvate ratio. However, Mintz and his colleagues used an incubation medium completely different from ours and from that used by Cartier and his colleagues.

1.2. Effect of glycolysis inhibitors

Addition of sodium monoiodoacetate (0.4 mmol) reduced lactate production by half but hardly changed pyruvate production (Fig. 1). The ATP level

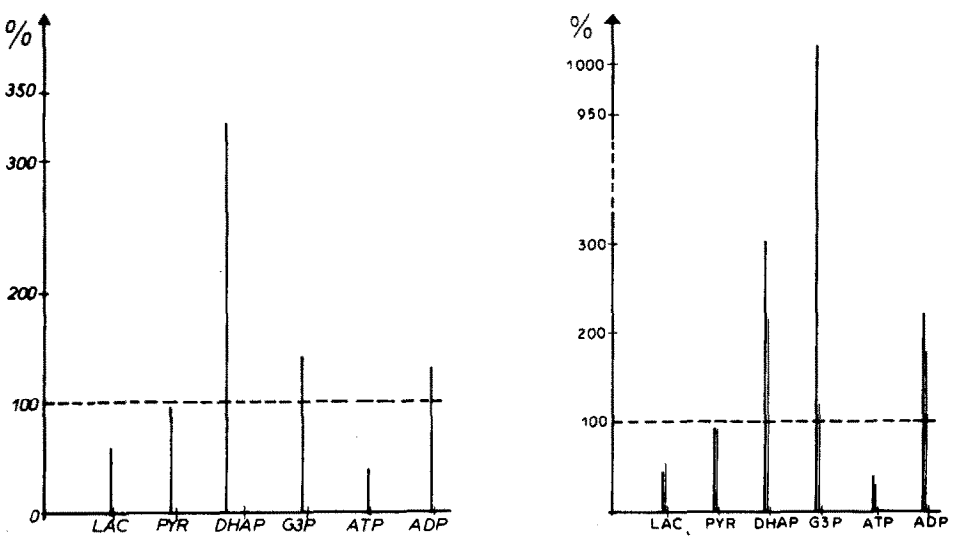

Fig. 1. Influence of sodium monoiodoacetate on glycolysis in human leukocytes. 80 X 10^6 to 100 X 10^6 cells were suspended in 1 ml glucose-free medium containing 0.4 mmol/l sodium monoiodoacetate and were incubated at 37°C for 60 min. The glycolysis metabolites were extracted and measured. The results are expressed as percentages of those found in the glucose-containing medium.

Fig. 2. Influence of sodium fluoride on glycolysis in human leukocytes. 80 X 10^6 to 100 X 10^6 cells were suspended in 1 ml glucose-free medium containing sodium fluoride (▬▬, 20 mM; ———, 5 mM) and were incubated at 37°C for 60 min. The glycolysis metabolites were extracted and measured. The results are expressed as percentages of those found in the glucose-containing medium.

was also markedly lowered, and the ATP/ADP ratio was 1.27. Similarly, sodium monoiodoacetate raised the level of DHAP but hardly affected G3P. This effect was apparently caused by inhibition of glyceraldehyde phosphate dehydrogenase, leading to a reduction in NADH regeneration and an accumulation of oxidated compounds. This inhibitor would also seem to shift glucose metabolism towards the glycerol 1-phosphate pathway [22], an active one for leukocytes [23]. Other authors have published results similar to these [7,8].

The effect of sodium fluoride is represented in Fig. 2. The two concentrations used decrease lactate and ATP production, whereas pyruvate production was not much affected. The ATP level was reduced by 2. This decrease in energy corresponds to a reduced glycolysis activity, the sodium fluoride decreasing oxygen consumption and thereby inducing an apparent Pasteur effect. The influence on the $NAD^+/NADH$ ratio varied with the sodium fluoride concentration. At 5 mmol/l, the dihydroxyacetone phosphate level was doubled and glycerol 3-phosphate did not vary significantly, whereas at 20 mmol/l, the level of the latter was multiplied by 10. This indicates a non-use of this metabolite in membranous phosphatides. The decrease in glycolysis activity seems to be caused by enolase inhibition. At high concentrations (20 mmol/l), sodium fluoride increases oxygen consumption and cAMP level by stimulating adenyl cyclase [7] but this effect has been contested [24].

Our results and those obtained by Marchand et al. [7] allow us to locate the regulation of the glycolytic pathway in leukocytes at the level of phosphofructokinase, hexokinase, and glyceraldehyde phosphate dehydrogenase.

2. Release of proteins and enzymes

2.1. Influence of glucose

We studied protein and enzyme release as a function of cell number, both with or without glucose (Fig. 3). The activities of PK, AP, and β-glucuronidase were proportional to cell number, demonstrating that enzyme activity in the ambient medium depended upon the activity in the pellet. However, LDH activity and the level of released protein did not vary directly with cell number, increasing until about 6×10^7 cells was reached and then remaining stable.

Other authors, working on chicken muscle [25], rate diaphragms, or other muscle [3,4] have obtained similar results. Some [3,4] found that this phenomenon occurred because the release of intracellular products seemed to be proportional to the surface/volume ratio of muscle fibers; for others [25], it depended upon the location of enzymes in relation to the cytoplasmic membrane; those far from the membrane would be less released. The more the number of cells is increased, the less surface contact there is with the medium. There seems to be a cell effect that would explain the plateau we found for proteins. We think that for LDH the phenomenon is due to the fact that certain isoenzymes would be linked more strongly to other cell constituents, namely to the nucleus [26,27]. The enzymes released can become bound again to the cells, fibroblasts [28], or organelles [29] from which they originated.

The percentages of enzyme and protein released in media 1 and 2 are represented in Table II.

Of the enzymes studied, AP was released in the highest percentage. This was

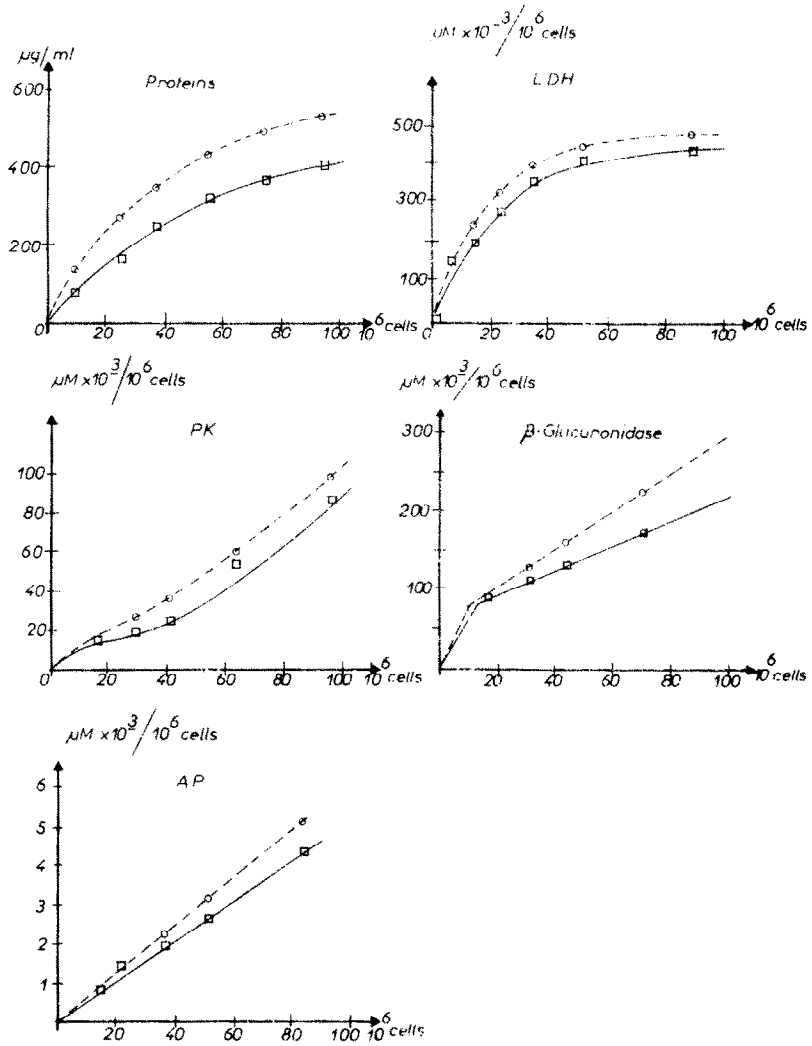

Fig. 3. Influence of the number of cells on the release of proteins and enzymes by human leukocytes. Various numbers of cells were suspended in 1 ml of either medium and incubated at 37°C for 60 min. The proteins and enzymes in the supernatants were measured after incubation and centrifugation for 10 min at 3000 rpm at +4°C. The results are expressed in $\mu M \times 10^{-3}/10^6$ cells for the enzymes and in µg/ml for the proteins. - - - - - -, Medium 1 (without glucose); ———, Medium 2, with glucose.

probably due to incomplete Triton X-100 extraction: at the detergent concentration used, we extracted 136 ± 20%, as did Avila and Convit [16] (110 to 140%), while Heralsi and Osserman [30], using no detergent, obtained 300–600% of the activity. β-Glucuronidase was the enzyme released in the lowest percentage in both media, reflecting greater stability of lysosomal structures.

The presence of a concentration of 16.5 mmol/l glucose in the medium reduced the amount of enzymes and total proteins released. The release of glycolysis enzymes was greatly lowered. The percentages found for LDH and PK were lower than those found by other authors working on human leukocytes or rat lymphocytes [31]. Thus, our experimental conditions seem to be

TABLE II

INFLUENCE OF GLUCOSE ON RELEASE OF TOTAL PROTEINS AND ENZYMES FROM HUMAN LEUKOCYTES

50×10^6 to 60×10^6 cells were suspended in 1 ml medium (1 or 2) and incubated at 37°C for 60 min. Proteins and enzymes were measured in the supernatants (S) and in the cellular pellets (C). The results are expressed in percentage of release ± the standard deviation.

	Total proteins	LDH	PK	β-glu	AP
Medium 1	4.76 ± 0.88	7.34 ± 1.35	5.55 ± 1.16	5.6 ± 1.03	10.25 ± 2.3
Medium 2 (with glucose)	3.41 ± 1.02	5.56 ± 1.07	2.24 ± 0.9	3.53 ± 0.8	7.37 ± 2.37
Percentage of decrease	−24	−25	−59	−36	−28

more favorable, the cells probably having a higher metabolic level. Glucose also had a distinct influence on β-glucuronidase, probably because of an increased stabilization of the lysosomal membrane. The effect of glucose on AP was less marked.

The influence of glucose was the same as that described for rat muscle [3,4,32,33] or perfused heart [1]. The consumption of glucose as an energetic substrate prevents the degradation of cell constituents, thereby maintaining a certain metabolic capacity. In addition, glycogen, which is not broken down, seems to stabilize the membranes [34].

2.2. Influence of glycolysis inhibitors

2.2.1. Effect of sodium monoiodoacetate. Results are presented in Table III.

Only PK was not increased in the presence of sodium monoiodoacetate. The degree of significance was 5%, except in the case of AP, where it was 10%. The decrease in PK can be explained by the enzyme's sensitivity to allosteric effectors [35,36] and to substrates other than ADP and fructose 1,6-diphosphate, which are accumulated when using this inhibitor [7], since the presence of substrates influences the release of PK [37].

The results obtained for the other metabolites were slightly different from

TABLE III

INFLUENCE OF GLYCOLYSIS INHIBITORS ON RELEASE OF PROTEINS AND ENZYMES FROM HUMAN LEUKOCYTES

50×10^6 to 60×10^6 cells were suspended with inhibitors in 1 ml medium (1 or 2) and incubated at 37°C for 60 min. The proteins and enzymes were measured in the supernatants (S) and the cellular pellets (C). The results were expressed as percentages of increase or decrease of results obtained in medium 1 compared with those obtained in medium 2.
Significance: * $p \leq 0.02$; ** $p \leq 0.05$; *** $P \leq 0.10$; N.S., not significant.

Inhibitors	Proteins	LDH	PK	β-glu	AP
Sodium monoiodoacetate (4 mM)	+30 **	+31 **	−38 **	+33 **	+32 ***
Sodium fluoride (5 mM)	+32 ***	+8 N.S.	+3 N.S.	+27 **	+50 **
Sodium fluoride (20 mM)	+97 *	+200 *	+493 *	+100 *	+230 *

those obtained by other authors. Zierler [33], working on rat muscle, found a 100% increase in aldolase release with a 10^{-4} M concentration of inhibitor; Englhardt [8], using leukocytes, measured a 200% LDH release, but with a 10^{-2} M concentration of inhibitor, a 3-h incubation period and a different survival medium than ours; and Bruns et al. [38] found an increase in serum LDH following injection of monoiodoacetate into animals. Monoiodoacetate decreases ATP and NADH production by inhibiting lipogenesis. These effects, taken individually or together, explain the enzyme release. ATP intervenes as an energy-giving substrate, but also intervenes on membrane. This compound decreases enzyme release induced by the phospholipases A and C [39]. Moreover, it maintains a balance between diffusible and non-diffusible proteins, and between protein aggregates or intracellular enzymes [40]. It is difficult to differentiate between these two effects.

2.2.2. Effect of sodium fluoride (Table III). The effect of this inhibitor varies with its concentration. At a 5 mM concentration, only the release of proteins and β-glucuronidase was significant, whereas at a 20 mM concentration, all the increases in release are significant at 2%. Our results are similar to those obtained by Englehardt [8].

At the same concentrations we have shown that sodium fluoride causes a decrease in energy production. This lack of energy would explain the enzyme release, at least at a 5 mM concentration. At high concentrations an accumulation of G3P is added to the lack of energy, and we no longer think that this latter compound is incorporated in membranal phosphatides. This phenomenon could explain the great enzyme release observed. Glycolysis would, then, play an important part in controlling enzyme release mechanisms in granulocytes, glucose being catabolized mainly by the Embden-Meyerhof pathway.

Conclusion

The results we obtained on human granulocytes demonstrate the existence of protein release control mechanisms similar to these obtained by authors using other materials, and demonstrate that granulocytes constitute and excellent model for in vitro study.

The decrease in energy production in all cases led to an increase in protein and enzyme release. However, this alone does not explain the phenomenon of enzyme release. In fact, the lack of energy was often associated with a decrease in or an inhibition of another metabolic pathway, e.g. lipogenesis, which is necessary to maintain membrane structures. For the leukocyte as well, metabolic integrity is a predominant factor in maintaining the cohesion of intracellular organization.

A change in intracellular energy results in a release of total proteins and enzymes into the extracellular medium. Such a mechanism can at least partially explain the origin of enzymes normally present in the plasma.

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