Increase of the glycolytic rate in human resting fibroblasts following serum stimulation

The possible role of the fructose-2,6-bisphosphate

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We report that glycolysis in human quiescent fibroblasts stimulated by serum addition is increased, and that the changes of the metabolic route reflect the activity of the phosphofructokinase. A possible role of fructose-2,6-bisphosphate as a positive modulator of the key enzyme is proposed.

Phosphofructokinase *Fructose-2,6-bisphosphate* *Glycolysis* *Human fibroblast*

1. INTRODUCTION

Glucose in the medium is the major source of energy for cultured cells: energy derives from the conversion of glucose to pyruvate and from its oxidation to CO_2 by the tricarboxylic acid cycle, although many cells derive most of their energy from glycolysis [1]. Glucose serves not only as source of energy but also of the compounds required for cell multiplication. Addition of growth factors to quiescent cells is known to enhance the glycolytic rate. This is attributable to an increased activity of phosphofructokinase (ATP:D-fructose 6-P-1 phosphotransferase, EC 2.7.1.11) (F6PK), the chief regulatory enzyme of glycolysis [2–4], rather than to an increased glucose transport.

We have therefore investigated F6PK activity in human fibroblasts during the initial phase of growth stimulation elicited by addition of fetal calf serum (FCS) to resting (serum-starved) cells.

Here, changes of F6PK levels assayed at different times after serum addition are described and evidence on the possible role of fructose-2,6-bisphosphate (Fru-2,6-P_2) as modulator of enzyme is presented.

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2. MATERIALS AND METHODS

Biochemicals and enzymes for the assay of F6PK, pyrophosphate:fructose-6-phosphate phosphotransferase (PP_i-PFK), lactate and glucose were from Boehringer (Mannheim). Other chemicals were purchased from Merck (Darmstadt). Fru-2,6-P_2 was kindly given by Dr E. Van Schaftingen. For the cell cultures Eagle's Minimum Essential Medium (MEM) and FCS (Gibco) were used.

2.1. *Cell cultures*

Human fibroblasts were obtained by biopsy from the forearm skin of normal subjects. Cultures were maintained in MEM supplemented with 10% FCS at 37°C in a humidified CO_2 incubator. For the experiments about 4×10^5 cells were seeded in 100-mm Petri dishes. At confluence the cultures were shifted to MEM containing 1% FCS to induce the so-called resting state. After 48 h of maintenance in serum-depleted medium,

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FCS was readded to a final concentration of 10%. A number of dishes was not treated with FCS, the cells were washed with PBS, scraped with rubber policeman and collected by centrifugation. The cells of the other dishes, enriched with FCS, were collected in the same manner at intervals of 30–240 min. At the selected times the culture medium was collected and assayed for the lactate [5] and glucose [6] content.

2.2. *Assay of F6PK in the cell extracts*

The pellet obtained from 5 dishes was resuspended with 300 μl Tris–HCl buffer 200 mM (pH 7.4) containing EGTA 0.5 mM, $MgCl_2$ 5 mM and 30% glycerol, sonicated with MSE-W ultrasonic disintegrator for 15 s and centrifuged in a Microfuge B for 3 min. The clear supernatant was used for the enzyme assay. The enzyme was assayed by measuring the disappearance of NADH spectrophotometrically at 340 nm at 30°C with a 2400 model Gilfor spectrophotometer. The routine mixture contained HEPES–NaOH 50 mM (pH 7.4), EDTA 0.2 mM, $MgCl_2$ 10 mM, NH_4Cl 1 mM, dithiothreitol 2.5 mM, ATP 2 mM, 0.4 units of desalted aldolase, 2.4 units of triosephosphate isomerase and 0.4 units of α-glycerophosphate dehydrogenase in 0.3 ml final vol. The cell extract was preincubated with the reaction mixture for 10 min and, when no background rate of NADH oxidation was recorded, the reaction was started by adding fructose-6-phosphate (Fru-6-P) 2.5 mM. One unit of F6PK is defined as the amount of the enzyme that catalyzes the formation of 1 μmol fructose-1,6-diphosphate/min at 30°C. The kinetic behaviour towards Fru-6-P was studied in the 0.1–10 mM range at 2 mM ATP. Protein content was determined as in [7], using bovine serum albumin as standard.

2.3. *Assay of Fru-2,6-P_2 in cultured fibroblasts*

For the determination of Fru-2,6-P_2, the pellet of one dish was mixed with 200 μl 0.05 N NaOH, heated for 5 min at 80°C, cooled and centrifuged. The supernatant was neutralized and aliquots were assayed for Fru-2,6-P_2 as in [8]. This method is based on the property of the Fru-2,6-P_2 to stimulate the PP_i-PFK prepared from potato tubers.

The amounts of Fru-2,6-P_2 were determined by comparing the increase of PP_i-PFK activation produced by the cell extracts with that produced by addition of known amounts of Fru-2,6-P_2 (0.5–5.0 pmol). An aliquot of each extract was subjected to acid hydrolysis for 10 min at 25°C (pH 1–2) to destroy Fru-2,6-P_2 and after neutralization it was tested for its ability to activate the PP_i-PFK.

3. RESULTS

The time-course of F6PK activity after addition of FCS to quiescent human fibroblasts indicates

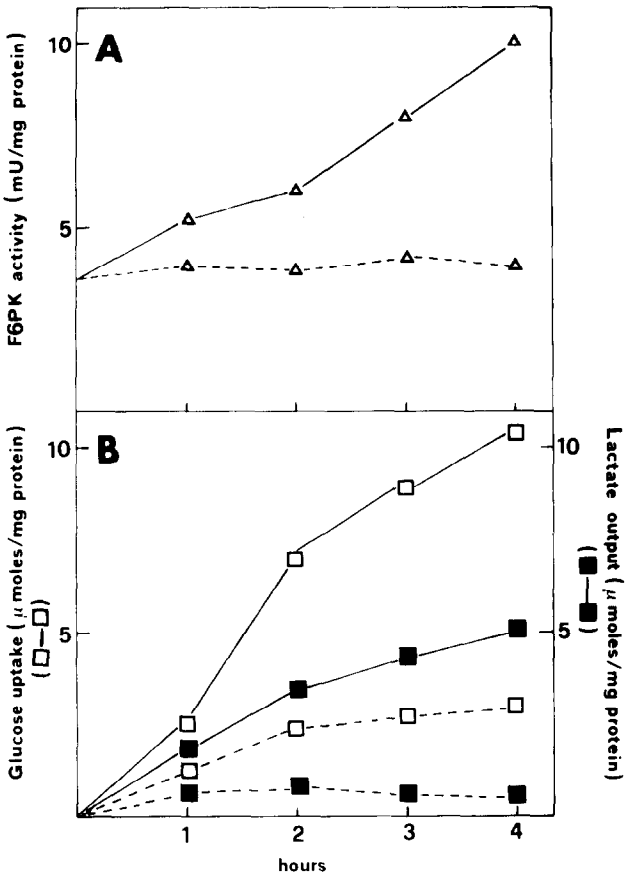

Fig.1. Effects of serum stimulation of human resting fibroblasts as a function of time. Serum-starved cultures were exposed to 10% FCS, and at the indicated times were assayed for F6PK activity (A), glucose uptake and lactate output (B). The dotted lines indicate the values of the same assays performed on unstimulated cells. Each point is the average of duplicate 100-mm dishes containing 5 ml of MEM.

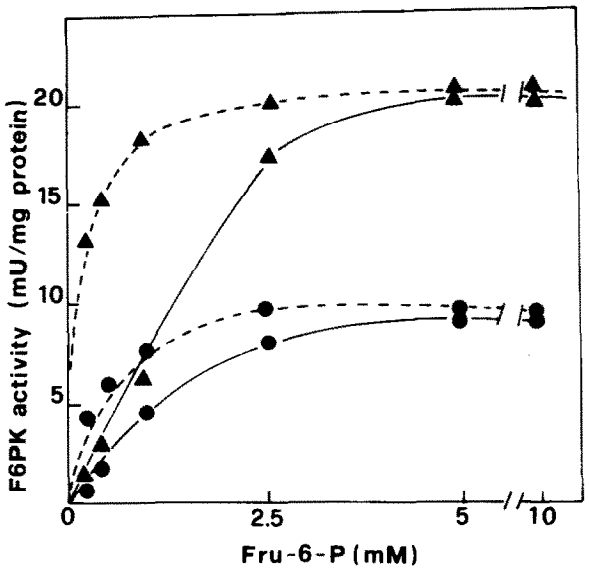

Fig.2. F6PK kinetics towards Fru-6-P in extracts of human quiescent fibroblasts unstimulated (●) and after 4 h of serum stimulation (▲). The dotted lines indicate the corresponding kinetics in the presence of Fru-2,6-P_2 1 μM. Mean values are derived from 3 or more individual determinations.

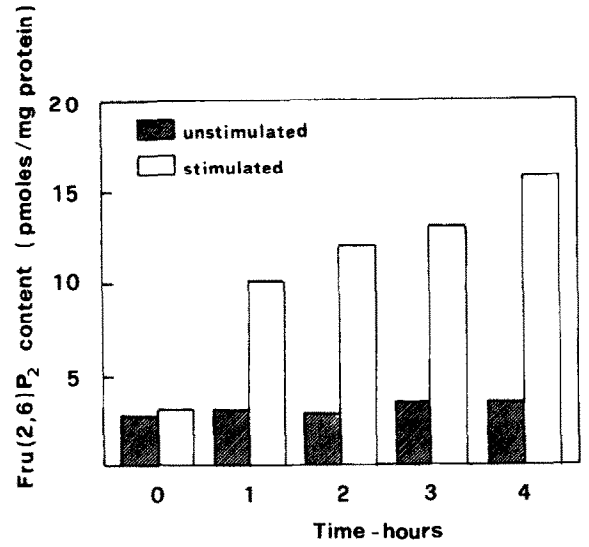

Fig.3. Effect of 10% serum addition on Fru-2,6-P_2 content of human resting fibroblasts as a function of time.

that at the fourth hour the enzyme reaches the highest level, about twice that of unstimulated cells (fig.1A). Glucose uptake and lactate production are in line with the enzyme pattern (fig.1B). Fig.2 shows the enzyme kinetics of the resting and stimulated cells as a function of [Fru-6-P] either in the presence of Fru-2,6-P_2 1 μM, or not. Addition of Fru-2,6-P_2 induces a marked increase of the affinity of F6PK for Fru-6-P: this effect is dose-related (not shown). Fig.3 shows the amount of Fru-2,6-P_2 detected at different times after the addition of FCS to the resting cells: the cell content of phosphate ester undergoes a rapid increase after stimulation, while it appears unchanged in unstimulated cells. Acid treatment of cell extracts abolished completely their ability to stimulate the PP_i-PFK.

4. DISCUSSION

The present results indicate that serum-stimulation of resting cells leads to an increase of glycolytic rate as shown by lactate output and glucose uptake. The activity of F6PK is increased within the first 4 h after serum addition. These findings are in accordance with those in [4], and confirm that the growth-related changes of glucose metabolism are due to an increase of F6PK activity rather than an increase of glucose transport. The independence of enzyme activation from glucose transport has been shown in similar experiments on 3T3 cells cultured in a glucose-free medium [3].

As to the mechanism of F6PK activation, Fru-2,6-P_2, as the most potent modulator of F6PK [9], would appear a likely mediator responsible for the activation of the enzyme in the stimulated fibroblasts. Fru-2,6-P_2 is present in various animal and plant tissues and as shown here this compound is present also in human fibroblasts. We have also observed that F6PK from both resting and stimulated fibroblasts exhibits marked sensitivity to Fru-2,6-P_2 although the fibroblast enzyme has been reported to be less susceptible than the muscle and liver isozymes [10]. Moreover, the time-course of the Fru-2,6-P_2 production in serum-stimulated fibroblasts suggests that it could play an important role in the enhancement of the glycolytic rate.

Since the exogenous Fru-2,6-P_2 fails to affect the V_{max} of fibroblast F6PK, the observed increase in enzyme activity in the stimulated cells cannot be

ascribed to the higher Fru-2,6-P_2 content. Association of inactive dimers to form active tetramers [11] or covalent modifications, such as phosphorylation and dephosphorylation of the enzyme molecule, may be responsible for the activation [12]. However, the increased content of Fru-2,6-P_2 in stimulated cells and its effect on the fibroblast F6PK affinity for Fru-6-P may account for the observed enhancement of the glycolytic rate.

These results suggest on the whole that Fru-2,6-P_2 could be the chemical signal that leads to the stimulation of glycolysis in cultured human fibroblasts. Although a clear connection between glycolysis and cell growth has not yet been established, it is tempting to speculate that Fru-2,6-P_2 may be involved in cell growth regulation. Markedly elevated cAMP levels were observed in cells made quiescent by serum restriction and an immediate fall in cAMP was shown when cells were released from quiescence [13]. cAMP prevents the formation and favours the destruction of Fru-2,6-P_2 [9], that in turn could counteract cAMP formation.

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REFERENCES

[1] Morgan, M.J. and Faik, P. (1981) Biosci. Rep. 1, 669–686.
[2] Diamond, I., Legg, A., Schneider, J.A. and Rozengurt, E. (1978) J. Biol. Chem. 253, 866–871.
[3] Schneider, J.A., Diamond, I. and Rozengurt, E. (1978) J. Biol. Chem. 253, 872–877.
[4] Fodge, D.W. and Rubin, H. (1973) Nature New Biol. 246, 181–183.
[5] Noll, F. (1974) in: L-(+)-Lactate: Determination with LDH, GPT and NAD (Bergmeyer, H.U. ed) Methods of Enzymatic Analysis, vol.3, 3rd edn, pp.1475–1479, Verlag Chemie; Academic Press, London, New York.
[6] Bergmeyer, H.U., Rubin, H., Schmidt, F. and Stork, H. (1974) in: D-Glucose. Determination with Hexokinase and Glucose-6-phosphate Dehydrogenase (Bergmeyer, H.U. ed) Methods of Enzymatic Analysis vol.3, 3rd edn, pp.1196–1201, Verlag Chemie and Academic Press, London, New York.
[7] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[8] Van Schaftingen, E., Lederer, B., Bartrons, R. and Hers, H.G. (1982) Eur. J. Biochem. 129, 191–195.
[9] Hers, H.G. and Van Schaftingen, E. (1982) Biochem. J. 206, 1–12.
[10] Meienhofer, M.C., Cottreau, D., Dreyfus, J.C. and Kahn, A. (1980) FEBS Lett. 110, 219–222.
[11] Bock, P.E. and Frieden, C. (1976) J. Biol. Chem. 251, 5630–5636.
[12] Brand, I.A. and Söling, H.D. (1975) FEBS Lett. 57, 163–168.
[13] Friedman, D.L. (1976) Physiol. Rev. 56, 652–708.