The effect of insulin on Fru-2,6-P_2 levels in human fibroblasts

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We report that fructose 2,6-bisphosphate (Fru-2,6-P_2) levels in quiescent human fibroblasts stimulated with insulin treatment increase in a time- and dose-dependent manner. The biochemical role of the sugar phosphate in the cellular response to the hormone which controls carbohydrate metabolism and acts as mitogenic factor is discussed.

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

1. INTRODUCTION

Fru-2,6-P_2 is a well-known sugar phosphate which has been demonstrated to be an important physiological regulator of carbohydrate metabolism and its concentration has been proved to be under hormonal regulation [1].

In [2] we showed the presence of Fru-2,6-P_2 in human fibroblasts; its concentration increases in quiescent fibroblastic cells stimulated to proliferate by the addition of serum indicated it as being the most potent growth-promoting factor.

The concomitant stimulation of glycolysis and of phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) (F6PK), one of the key enzymes of this metabolic pathway, was also observed as an early metabolic response induced by serum.

Among the many different factors which control the growth of mammalian cells, insulin is a well-known growth-promoting substance [3]. Although it is a poor mitogen, at least for mammalian cells, its role in cell division is far from negligible since insulin potentiates the effect of other growth factors like EGF in a number of cell systems.

Insulin has a marked effect on carbohydrate metabolism, which is evidenced by enhanced uptake of glucose [4], increased rate of glycolysis [4] and augmented activity of key glycolytic enzymes [5,6]; these are effects very similar to those elicited by serum. In this connection it appears reasonable to investigate whether the polypeptide hormone added to resting fibroblasts can modify the intracellular concentration of Fru-2,6-P_2 as has been reported for serum treatment.

2. MATERIALS AND METHODS

Biochemicals and auxiliary enzymes for the assay of Fru-2,6-P_2 and glucose were purchased from Boehringer Mannheim GmbH (Heidelberg). Bovine pancreas insulin and epidermal growth factor (EGF) were obtained from Sigma (St Louis, MO). Fru-2,6-P_2 was kindly provided by Dr E. van Schaftingen. For the cell cultures Eagle's minimum essential medium (MEM), phosphate-buffered saline (PBS) and fetal calf serum (FCS) from Gibco were used.

2.1. *Cell cultures*

Human fibroblasts were obtained by biopsy from the forearm skin of normal subjects. Cell cultures were maintained in MEM supplemented with 10% FCS at 37°C in a humidified atmosphere of 95% air, 5% CO_2. For the experiments about 1.5×10^5 cells were seeded in 60-mm petri dishes. At confluence the cultures were shifted to MEM-containing 1% FCS to induce the resting state. After 3 days of maintenance in serum-depleted medium, the monolayers were used for ex-

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periments: the test substances were added in a small volume of aqueous 0.1% (w/v) solution of chicken ovalbumin to the cell cultures without changing the medium. At selected times the culture medium was collected and assayed for glucose content [7].

2.2. *Assay of Fru-2,6-P_2*

For the extraction of Fru-2,6-P_2, monolayers were rapidly washed with PBS and 500 μl of hot 50 mM NaOH were added directly to each dish. This extraction procedure gives higher values of Fru-2,6-P_2 than those obtained with the previous method [2]. The extracts were quickly transferred to stoppered tubes and heated for 10 min at 80°C. Fru-2,6-P_2 content of the extracts was assayed as in [8] based on the property of Fru-2,6-P_2 to stimulate the pyrophosphate: fructose-6-phosphate 1-phosphotransferase (EC 2.7.1.90) (PP_i-PFK) prepared from potato tubers [9]. The amounts of Fru-2,6-P_2 were determined by comparing the increase of PP_i-PFK activity produced by cell extracts with that produced by known amounts of Fru-2,6-P_2. The commercial fructose 6-phosphate routinely used as substrate in the assay was acid-treated to destroy traces of contaminant Fru-2,6-P_2 [8]. Protein content was determined with the Coomassie blue method using bovine serum albumin as standard [10].

The data are presented as means of values from triplicate assays in a representative experiment. All observations have been repeated at least twice in separate experiments with different fibroblast strains.

3. RESULTS

Human resting fibroblasts were incubated for different times and their Fru-2,6-P_2 content was determined in the absence or presence of 100 nM insulin. The data reported in fig.1 show that, upon exposure to insulin, a progressive increase of the intracellular content of the sugar phosphate was detected: after 7 h incubation an approx. 2-fold increase was observed. Fig.2 shows the dose-response curve for the insulin-induced increase of Fru-2,6-P_2 content measured in quiescent fibroblasts after 7 h exposure to the hormone: the half-maximal effect is obtained at about 6 nM insulin;

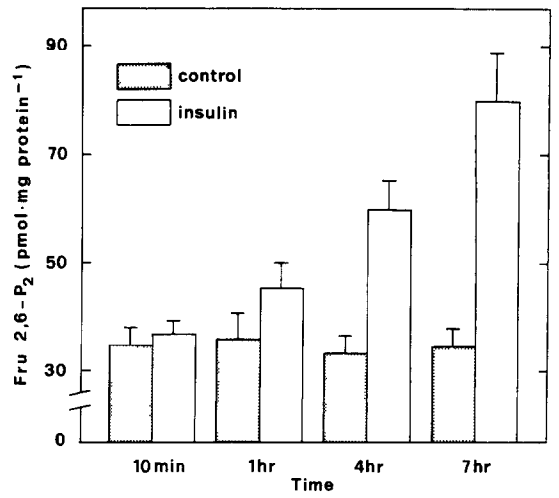

Fig.1. Effect of 100 mM insulin addition on Fru-2,6-P_2 content of human resting fibroblasts as a function of time. Controls received 0.1% (w/v) ovalbumin alone. Each value is the average of triplicate dishes.

hormonal concentrations higher than 100 nM give no further increase in Fru-2,6-P_2 content. In the same figure is reported the effect of the same range of concentrations of insulin on cellular glucose consumption. The resulting pattern indicates that, during the incubation period, the hormone is able

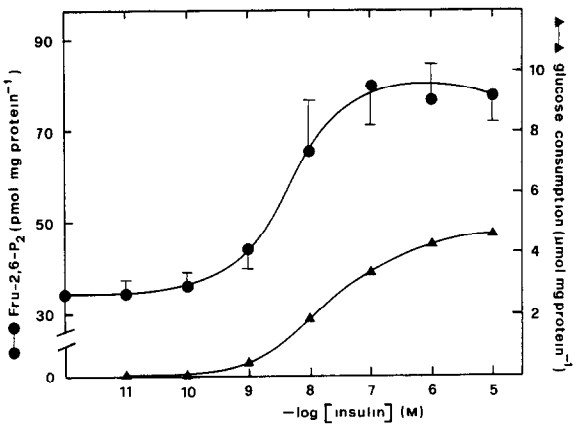

Fig.2. Dose-response curve of Fru-2,6-P_2 levels with varying concentrations of insulin in human resting fibroblasts. After the addition of insulin the cells were incubated for 7 h and then assayed for Fru-2,6-P_2. At each point the collected medium was assayed for glucose content. Values shown are means ± SE of experiments performed in triplicate.

Table 1

Effect of insulin and EGF on Fru-2,6-P_2 content in human fibroblasts

Addition	Fru-2,6-P_2 (pmol/mg protein)
None	35 ± 2
Insulin (10^{-7} M)	80 ± 8
Insulin (10^{-6} M)	73 ± 7
EGF (10^{-6} M)	66 ± 2
Insulin (10^{-7} M) plus EGF	82 ± 5
Insulin (10^{-6} M) plus EGF	90 ± 6

Experimental conditions were the same as reported in fig.2

to stimulate carbohydrate metabolism in a dose-related manner.

The effect of EGF, another well-known growth-promoting factor on Fru-2,6-P_2 content, has been examined using the same resting fibroblasts: the experimental conditions and results are reported in table 1. EGF (1 μM) also increases the intracellular concentration of sugar phosphate and an additional increase is observed with both insulin and EGF.

4. DISCUSSION

The relationship between insulin and Fru-2,6-P_2 levels in rat hepatocytes has been extensively investigated [11,12], and the hormonal effect appears to be the result of counteracting the action of glucagon. So far no evidence has been reported on the effectiveness of insulin by itself in enhancing Fru-2,6-P_2 levels.

Our results indicate that insulin stimulation of human resting fibroblasts leads to a clear increase in intracellular Fru-2,6-P_2 content. Moreover, the enhancement of sugar phosphate concentration, as an effect of insulin treatment, is dose-dependent: from the dose-response curve it appears that physiological concentrations of insulin are ineffective in raising Fru-2,6-P_2 levels, whereas maximal increase is obtained with concentrations far in excess of physiological ones.

On the other hand, very high insulin concentrations are required to stimulate appreciably DNA synthesis in density inhibited fibroblasts [13,14] through a number of molecular events that are still largely unknown. The fact that high concentrations of insulin are required to elicit a significant increase either in Fru-2,6-P_2 or in DNA synthesis must not be underestimated: the ineffectiveness of physiological concentrations of insulin in exerting these biological effects could be due to a significant change in the physico-chemical characteristics of insulin receptors in vitro as proposed in [15]. Another interesting finding of this report is that the effect of EGF on Fru-2,6-P_2 content is similar to that produced by insulin and the action of insulin is partially additive to that exerted by EGF; the latter result may suggest a common molecular mechanism which controls sugar phosphate levels.

In summary, our results show a novel cellular response to insulin action which at least accounts for the well-established increase in glycolytic flux elicited by the hormone, likely as a consequence of the positive modulation of fibroblast F6PK by Fru-2,6-P_2. However, we are tempted to speculate that the enhancement of the sugar phosphate may represent a biochemical feature associated with cell proliferation; this hypothesis is supported by our recent research (in preparation) that shows higher Fru-2,6-P_2 levels in proliferating cells compared with confluent ones without stimulation of glycolysis.

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