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INCREASE OF FRUCTOSE-1,6-DIPHOSPHATASE ACTIVITY IN CULTURED HUMAN PERIPHERAL LYMPHOCYTES AND ITS SUPPRESSION BY PHYTOHEMAGGLUTININ

Wang-F. Fong,* Ivo Hynie,** Leo Lee** and J.B. Ralph McKendry*

*Division of Metabolism and Endocrinology, Ottawa Civic Hospital, Ottawa, Canada, K1Y 4E9 and
**Laboratory Center for Disease Control, National Health & Welfare, Ottawa, Canada, K1A 0L2

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Summary: The specific activity of fructose-1,6-diphosphatase in freshly isolated human peripheral lymphocytes is usually less than 10% of what can be found in normal animal livers. The enzyme activity is strongly inhibited by AMP and is also inhibited by its substrate, fructose-1,6-diphosphate, at concentrations higher than 20 μM. In cultured lymphocytes the enzyme activity gradually increases so that by day 3 of the incubation the specific enzyme activity could be 15 to 80 fold higher than the preincubation level. The increase would continue for at least 10 days if the culture medium is periodically renewed. When phytohemagglutinin is present in the culture medium, the increase of the enzyme activity is completely suppressed. No free soluble enzyme inhibitor could be detected in phytohemagglutinin-treated cells by mixing and dialysis experiments.

Blood cells are generally regarded as glucose consuming tissues and in many instances it has been assumed that they contain no fructose-1,6-diphosphatase (D-fructose-1,6-bisphosphate 1 phosphohydrolase, EC 3.1.3.11) a key gluconeogenic enzyme. Nevertheless a small but significant amount of fructose-1,6-diphosphatase activity has been detected in leukocytes (1) and particularly in lymphocytes (2). The specific enzyme activity in freshly isolated lymphocytes is usually no more than 10% of what can be found in normal animal livers.

In a previous report we have shown that an oral glucose load would cause fluctuations in the fructose-1,6-diphosphatase activity in peripheral lymphocytes (2). Furthermore, at the third hour after an oral glucose load the enzyme activity in lymphocytes isolated from normal subjects would be comparable to the fasting level whereas in cells isolated from diabetics the enzyme activity was significantly elevated (2). Alimentary hyperglycemia does not inhibit hepatic gluconeogenesis in mice (3) and following glucose administration accelerated

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incorporation of gluconeogenic precursors into glycogen has been observed both in rat hepatocytes in vitro (4) and in human and rat liver in vivo (J. Radziuk, personal communication). The reason for this apparently paradoxical increase of gluconeogenesis in the face of an over abundance of glucose and the mechanism regulating this response are not known.

In order to isolate the factor(s) affecting fructose-1,6-diphosphatase in lymphocytes, we have been studying the enzyme activity in cultured cells. Here we wish to report the progressive increase of the enzyme activity in lymphocyte cultures and the suppression of this increase by the presence of the mitogen PHA[1] in the culture medium.

METHODS

Human venous blood samples were collected from apparently healthy volunteers who had no family background of diabetes mellitus. Disodium EDTA was added at a concentration of 1.4 mg/ml as anticoagulant. Lymphocytes were isolated using Ficoll-Paque (trademark of Pharmacia) following the procedure suggested by the manufacturer and phosphate-buffered saline was used for the dilution of blood and the washing of cells. Final lymphocyte preparations contained less than 5% other white cells and platelets and were practically free from erythocytes. Over 97% of the lymphocytes excluded trypan blue.

Lymphocytes were cultured in medium 199 (Gibco) containing 100 units per ml each of penicillin and streptomycin and supplemented with 10% decomplemented, pooled human AB serum. Each culture contained, in 16 x 125 mm culture tube, 2 to 5 million cells in 2 ml of medium and was incubated at 37°C with 5% CO_2 in air as the gas phase. PHA-M (Difco lot # 620793) was used at a final concentration of 1% v/v. At this concentration of the mitogen the Stimulation Index (tritiated thymidine incorporation during the last 16 hours of a stimulated 3 day culture/similar value obtained from a non-stimulated culture) was consistently in the range between 50 and 200.

For harvesting, excess culture medium was siphoned off and cells were washed once with cold TEM saline (100 mM Tris, pH 7.2; 5 mM $MgSO_4$; 0.1 mM disodium EDTA and 50 mM NaCl) and resuspended in TEM buffer (same as TEM saline but without NaCl) at a final density of approximately 10 x 10^6 cells/ml. Cells can be stored frozen at -20°C for up to 2 weeks without any noticeable loss of the enzyme activity.

To prepare cell extracts, frozen-thawed samples were sonicated for 2 bursts of 10 seconds to break up clumps and the total protein was determined in crude homogenates by the Lowry's method (5). Fructose-1,6-diphosphatase in supernatant fractions obtained by centrifuging crude homogenates at 20 000 g for 30 minutes was measured essentially according to the spectrophotometric method of Pontremoli et al (6). The assay mixture totalling 0.3 ml contained, in TEM buffer, 2 I.U. of glucose-6-phosphate dehydrogenase, 4 I.U. of phosphoglucose isomerase, 0.5 mM of $NADP^+$ and up to 50 units of fructose-1,6-diphosphatase. One unit of enzyme activity is defined as one nmole $NADP^+$ reduced per minute. The mixture was incubated at 37°C for 5 minutes and background change at 340 nm was recorded. The reaction was then started by the addition of fructose-1,6-diphosphate to a final concentration of 20 µM.

[1] Abbreviation : PHA, phytohemagglutinin.

RESULTS

General properties of human lymphocyte fructose-1,6-diphosphatase

Human lymphocyte fructose-1,6-diphosphatase shares common properties with enzymes from other sources. The enzyme activity in dialyzed crude cell extracts is strongly inhibited by AMP and is also inhibited by the substract, fructose-1, 6-diphosphate, at concentrations higher than 20 μM (Figure 1, a and b). However, the inhibition by fructose-1,6-diphosphate is never more than 40%. The optimum pH for the enzymatic reaction is around neutral and for the present work the enzyme is routinely assayed at 37°C, pH 7.2.

In our experience, the specific activity of fructose-1,6-diphosphatase in freshly isolated lymphocytes was usually less than 10% of what can be found in animal livers which normally contain around 100 units per mg protein.

Increase of fructose-1,6-diphosphatase activity during culture

During in vitro culture, the fructose-1,6-diphosphatase activity in lymphocytes would progressively increase. Although the degree of increase could vary by several fold from experiment to experiment, it has been our experience that by the end of the third day of the incubation the enzyme activity could be anywhere from 15 to 80 fold higher than the preincubation level. The enzyme activity continued to steadily increase until day 5 and could further increase if the culture medium was renewed (Figure 2). Renewing the culture medium every day had only positive effect on the increase of the enzyme activity which suggested that the increase was not caused by the accumulation of metabolites in the culture medium. With the renewal of the culture medium the increase of the enzyme activity could continue for at least 10 days. Cultures older than 10 days were not studied due to the lack of knowledge concerning such old cultures and their tendency to deteriorate.

Effect of PHA

PHA, a glycoprotein isolated from the red kidney bean *Phaseolus vulgaris*, is a nonspecific mitogen for lymphocytes. Stimulation of lymphocyte glycolysis and Kreb's cycle activity by PHA is well documented (e.g. 7, 8, 9 and 10).

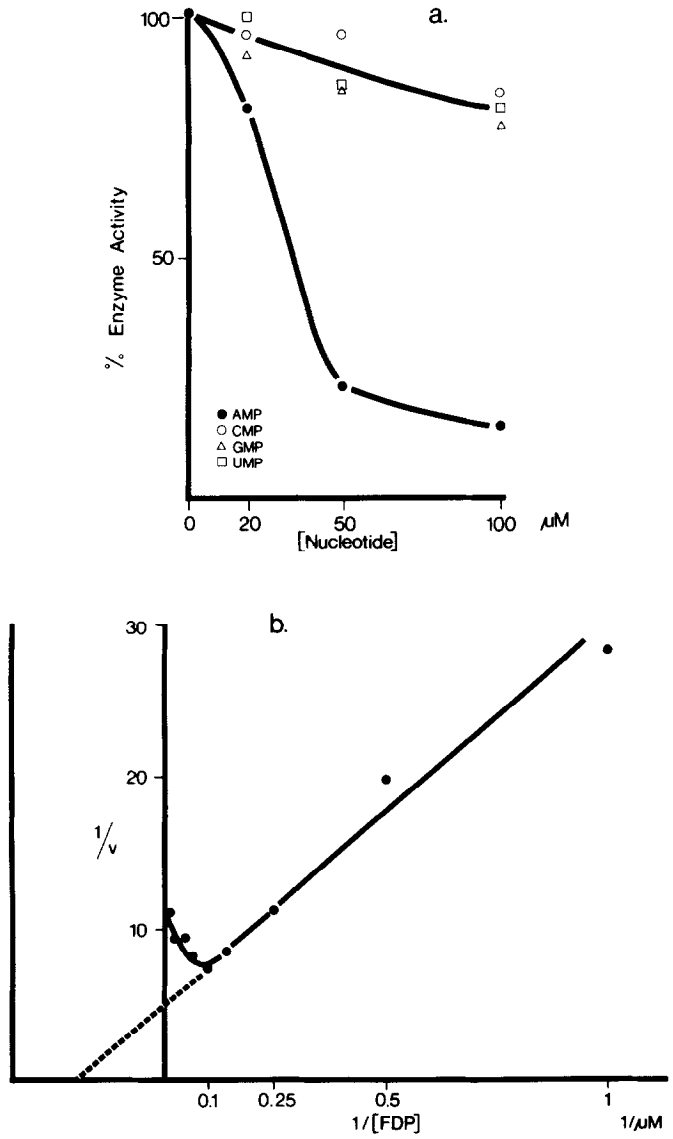

Figure 1. a) The inhibition of human lymphocyte fructose-1,6-diphosphatase by AMP. b) Lineweaver-Burk plot of the reciprocal of reaction rate (1/nmol $NADP^+$ reduced per min) versus the reciprocal of fructose-1,6-diphosphate concentration (1/μM). Fructose-1,6-diphosphatase activity in dialyzed crude cell extracts was assayed as described in METHODS. Each assay contained approximately 0.2 unit of enzyme. Nucleotides and fructose-1,6-diphosphate were added to enzyme assay mixtures at concentrations indicated.

The increase of fructose-1,6-diphosphatase activity in lymphocyte cultures was completely suppressed by the presence of PHA in the culture medium (Figure 2). At the concentration of PHA used the lymphocyte cultures responded to the mito-

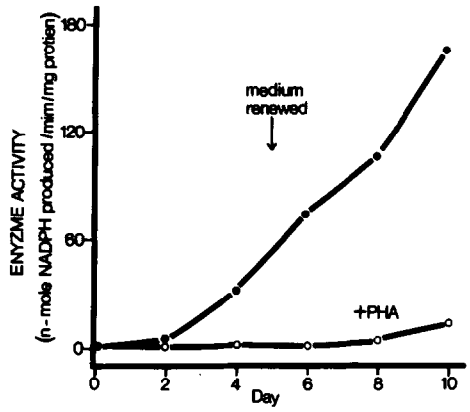

Figure 2. The increase of fructose-1,6-diphosphatase activity in human lymphocyte cultures and its suppression by PHA. Culture conditions were detailed in METHODS and PHA was present in treated cultures throughout the experiment. Media in both control and PHA-treated cultures were renewed on day 5 of the incubation. The specific enzyme activity at the beginning of the incubation was 1.05 units per mg protein (1 unit = 1 nmole NADPH produced per minute).

genic stimulation normally as indicated by the stimulated incorporation of tritiated thymidine (see Methods). PHA added directly to enzyme assay mixtures at various concentrations up to the concentration used in lymphocyte cultures did not have any detectable effect on the enzyme activity.

The suppression of fructose-1,6-diphosphatase activity in PHA-treated lymphocytes was probably not caused by the presence of free soluble inhibitory substance(s) in cell extracts. Extensive dialysis for 48 hours against two changes of 1 000 volume of buffer did not increase the enzyme activity in extracts of PHA-treated cells. Furthermore, adding extracts prepared from PHA-treated cells to extracts prepared from non-treated cells did not cause any inhibition to the enzyme activity in the latter.

DISCUSSION

Fasting and/or postprandial hyperglycemia is one of the clinical manifestations of mature onset diabetes and there is very little doubt that it also contributes to the development of other diabetic complications. Although the absolute rate of splanchnic glucose production in diabetics with fasting hyperglycemia is comparable to normal, the contribution of gluconeogenic precursors

to the glucose output in these diabetics is about twice of that of normal individuals (11). Our aim is to understand the mechanism regulating gluconeogenesis and eventually apply our knowledge to the diagnosis and treatment of diabetes mellitus.

In this communication we report the first time the progressive increase of fructose-1,6-diphosphatase activity in cultures of human peripheral lymphocytes. At present we do no know which, if there is any, factor(s) in the culture medium is particularly essential to the increase. However, cultured lymphocytes do synthesize glycogen with or without PHA stimulation (8) and we believe gluconeogenesis contributes to this process.

It is well documented that PHA stimulates glycolysis in cultured lymphocytes (e.g. 7, 8, 9 and 10). In non-stimulated cells certain glucose catabolic enzymes (lactate dehydrogenase, glucose-6-phosphate dehydrogenase) tend to decrease over time (12). Our findings are well in line with these observations.

A number of factors in the blood circulation may cause an increase in the rate of gluconeogenesis. These include nutritional factors, e.g. gluconeogenic precursors and glucose (4), as well as hormonal factors, e.g. glucagon. Direct and indirect evidence has suggested that the activation of fructose-1,6-diphosphatase is at least partly responsible for the glucagon-stimulated gluconeogenesis in rat liver (13, 14 and 15). Thus it is of great interest to note that fructose-1,6-diphosphatase might be activated by phosphorylation through the action of cyclic AMP-dependent protein kinase (16). Our research is now moving along these lines and cellular effects of nutritional and hormonal factors on the regulation of fructose-1,6-diphosphatase activity in diabetics and normal individuals is under study.

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