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THE SHIFT OF AN INCREASE IN PHOSPHOFRUCTOKINASE ACTIVITY FROM PROTEIN SYNTHESIS-DEPENDENT TO -INDEPENDENT MODE DURING CONCANAVALIN A INDUCED LYMPHOCYTE PROLIFERATION

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SUMMARY: Phosphofructokinase activity increased dramatically in cultured mouse spleen lymphocytes 8 hours after concanavalin A stimulation and preceded the onset of DNA synthesis by 8 hours. The increase in enzyme activity and [^{3}H]-thymidine incorporation were mitogen-concentration dependent. Enzyme activity increased 12-fold over control level at 48 hours when DNA synthesis peaked. The protein synthesis inhibitor, cycloheximide, blocked the rise in phosphofructokinase only when given prior to the increase in enzyme activity. Once the increase began, later addition of cycloheximide became progressively less inhibitory. These observations suggest that the period of increase in phosphofructokinase activity involves the activation of preexisting enzyme molecules.

INTRODUCTION: Aerobic glycolysis in lymphocytes increases more than 50-fold during Con A** induced mitogenesis (1). We found the increase in glycolysis to be associated with cell proliferation; more specifically, it is temporally related to DNA synthesis (1). To further understand these changes in the regulation of glucose metabolism, we have studied the behavior of PFK (E.C. 2.7.1.11), a key regulatory enzyme in the glycolytic pathway, in proliferating lymphocytes. In this communication, we show activation of PFK by Con A in cultured lymphocytes takes place several hours before the onset of DNA synthesis and is independent of protein synthesis once the process begins.

MATERIALS AND METHODS: Chemicals: Chemicals and enzymes were purchased from Sigma Chemical Co. [^{3}H]-Tdr and [^{3}H]- Leu were from New England Nuclear Co.

Lymphocyte Cultures: Lymphocytes from mouse spleens were isolated and cultured as described by Wang et al (1). DNA and protein synthesis were determined by the incorporation of [^{3}H]-Tdr and [^{3}H]-Leu into trichloroacetic precipitable material during a one hour pulse period (1).

Preparation of Lymphocyte Extracts and Assays of PFK: Lymphocytes were washed with saline and suspended in 10 mM tris buffer, pH 7.5, containing 30 mM potassium fluoride, 4 mM EDTA, 2 mM dithiothreitol and sonicated 3 times for 5

** Abbreviations: CHX, cycloheximide; Con A, concanavalin A; ^{3}H-Leu, ^{3}H-leucine; PFK, phosphofructokinase; and ^{3}H-Tdr, ^{3}H-thymidine.

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seconds with a Bronson sonifier. The sonicate was centrifuged at 10,000 x g for 20 minutes; the supernatant was used in assaying PFK activity. Protein content was determined by the method of Lowry et al (2) with bovine serum albumin as a standard. PFK activities were determined by measuring the disappearance of NADH spectrophotometrically. Except where indicated otherwise, the enzyme activities were measured at pH 8.2 under optimum conditions as described by Tsai and Kemp (3). One unit of enzyme converts 1 umole of fructose-6-P to fructose-1, 6-diphosphate at pH 8.2 in one minute. Assays of PFK activity at pH 7.4 were also done as previously described (3).

RESULTS: The changes in PFK activity in cell extracts derived from lymphocytes during the proliferative response to Con A are shown in Table 1. Similar results were obtained whether the enzyme activities were calculated as a function of cell number or cell protein content. PFK activity in resting, unstimulated lymphocytes was low and stayed relatively constant for the entire culture period. Lymphocytes stimulated by Con A, however, showed a dramatic increase in PFK activity over control levels; peak activity appeared about or just after the period of maximum DNA synthesis (48 hours after Con A addition). PFK activity, moreover, remained high 96 hours after Con A stimulation by which time [^{3}H]-Tdr incorporation had waned.

A more detailed time course of the changes in PFK activity is shown in Fig. 1. PFK activity did not begin to increase over the basal level until 8 hr after Con A stimulation, but rose rapidly thereafter. Compared to the time course of [^{3}H]-Tdr incorporation, the increase in PFK activity preceded the onset of DNA synthesis by approximately 8 hrs. This observation established that the increase in

TABLE ONE

The Activity of PFK and the Rate of [^{3}H]-Tdr Incorporation in Mouse Spleen Lymphocytes Cultured in the Absence or the Presence of 1 ug/ml Concanavalin A.

Culture Time (hours)	PFK activity, pH 8.2 (units/10^8 viable cells)		(units/mg protein)		[^{3}H]-Tdr incorporation (cpm/10^6 viable cells/hr)	
	-Con A	+Con A	-Con A	+Con A	-Con A	+Con A
24	0.036	0.120	0.013	0.021	885	18,290
48	0.024	0.347	0.012	0.052	915	45,960
72	0.036	0.420	0.013	0.075	1,720	20,750
96	0.046	0.360	0.014	0.070	2,132	11,570

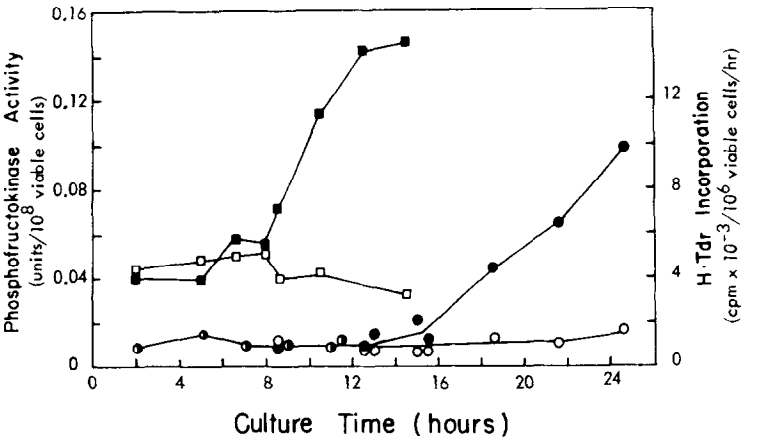

Fig. 1. Time courses of PFK activity and [^{3}H]-Tdr incorporation in cultured mouse spleen lymphocytes. Lymphocytes were cultured in the absence (open symbols) or the presence of 1 ug/ml Con A (closed symbols) in RPMI 1640 medium supplemented with 1% heat-inactivated human serum, 2 mM L-glutamine, 50U/ml penicillin and 50mg/ml streptomycin. At time points indicated, samples were assayed for PFK activity (□,■) and [^{3}H]-Tdr incorporation (○,●). The results were derived from three independent experiments.

PFK enzyme activity was a pre-S phase event in Con A stimulated lymphocytes. Moreover, in terms of PFK activity, the pre-S phase can be further divided into an early period of low PFK activity (0-8 hrs) and a late period of rapidly rising enzyme activity (8-16 hrs).

The association between the increase in PFK activity and lymphocytes proliferation was further strengthened by determining the effect of varying degrees of Con A stimulation. Lymphocytes were cultured with differing concentrations of Con A for 48 hr then assayed for PFK activity and DNA synthesis. The increase in [^{3}H]-Tdr incorporation and PFK activity in the extracts showed a parallel, biphasic response (Fig. 2). The activity of PFK increased rapidly over control level with increasing concentrations of Con A, peaked between 1 and 2 ug/ml then diminished progressively with higher doses of the mitogen. The striking similarity between the responses of PFK activity and [^{3}H]-Tdr incorporation to Con A stimulation suggests that the increase in PFK activity is an integral feature of lymphocyte proliferation.

It is well known that, at physiological pH, the activity of PFK is under the influence of many allosteric effectors (4). This control is largely abolished

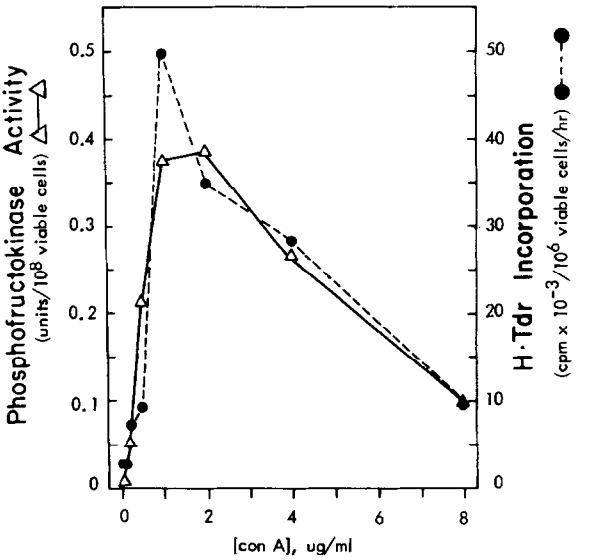

Fig. 2. Activity of PFK and the rate of [^{3}H]-Tdr incorporation in Con A stimulated lymphocytes. Lymphocytes were cultured with various concentrations of Con A for 48 hours; samples were taken from the cultures for assaying PFK activity (△—△) and the incorporation of [^{3}H]-Tdr (●--●).

at alkaline pH. Therefore, the activation of PFK, observed in cell extracts assayed at pH 8.2 under optimal conditions, is unlikely to be due to the presence of allosteric effectors. Extracts of unstimulated and Con A stimulated lymphocytes were also assayed for PFK activity at pH 7.4. A similar increase in PFK activity in extracts of Con A stimulated cells over control levels was observed (data not shown). The enzyme activity in all extracts showed a sigmoidal saturation curve for fructose-6-phosphate and an increase in sensitivity to ATP inhibition when the reaction pH was decreased from 8.2 to 7.4. Consequently, the increased PFK activity in lymphocytes after Con A stimulation was not caused by the loss of allosteric regulation.

To determine whether or not the increase in PFK activity resulted from an increase in the synthesis of new enzyme molecules, we used CHX, a protein synthesis inhibitor. At different time points after Con A stimulation, CHX (2 ug/ml cultures) was added to lymphocyte cultures, a dosage which blocked completely the incorporation of [^{3}H]-Leu. The activity of PFK in these cultures was measured after various lengths of incubation with the inhibitor. Our results

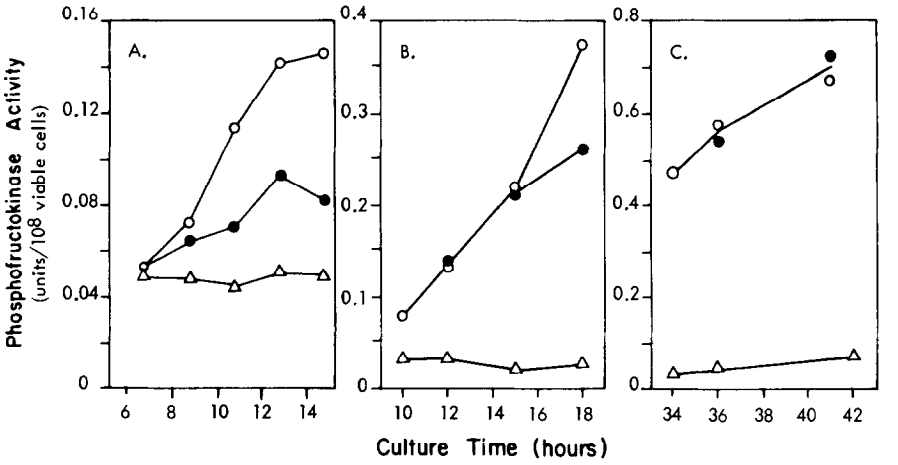

Fig. 3. The effect of CHX treatment on the increase of PFK activity in Con A stimulated lymphocytes. Lymphocyte cultures were set up as described in the legend for Fig. 1. At (A) 6.5 hours, (B) 10 hours and (C) 34 hours after Con A stimulation, CHX was added to the cultures. At the indicated time points, PFK activity in extracts from lymphocytes cultures without Con A (△), with Con A (○), and with Con A and CHX (2ug/ml) (●) was determined.

again showed the pre-S phase could be divided into two periods. The rise in PFK activity could only be effectively blocked when CHX was added in the early G_1 phase before the increase began. The period of PFK activity rise itself in late G_1 phase was not sensitive to CHX inhibition.

Specifically, the addition of CHX to lymphocytes within 5 hr of Con A stimulation completely blocked the later increase in PFK activity. If the CHX addition was made at 6.5 hr, or just prior to the onset of increase in PFK activity, an intermediate effect was seen (Fig. 3A). The rise in enzyme activity began at the same time but proceeded at a slower rate than that in untreated cells. When the addition was delayed until 10 hr after Con A stimulation, or shortly after the change in PFK activity began, the increase continued normally for 5 hr (Fig. 3B). The slight fall off with longer periods of inhibition presumably reflects the degree of asynchrony associated with Con A stimulation (5). Finally, if CHX addition was further postponed until 34 hr after Con A, the increase in PFK activity was totally insensitive to the inhibitor for as long as 8 hr (Fig. 3C).

DISCUSSION: The sharp increase in PFK activity accompanying cell proliferation reported here is consistent with our previous finding that marked aerobic gly-

colysis occurs in normal dividing lymphocytes (1). The rise in activity of PFK as well as two other key glycolytic enzymes, lactate dehydrogenase and pyruvate kinase (Wang, Abbsnezhad, Yesmineh and Foker, in preparation) begins several hours prior to the onset of DNA synthesis. Lactate production, however, began only at S phase (1). These observations indicate a pre-S phase increase in glycolytic enzymatic capacity occurs in preparation for the burst of glycolysis during S phase. About 8 hrs after Con A stimulation lymphocytes appear to undergo an important transition and become committed to proliferation (6). So it is of particular interest that at this same time, glycolytic enzyme activation began.

Increased PFK activity has also been found during proliferation of other cultured cells (7-9) and has been postulated to be responsible for the elevated aerobic glycolysis observed in these dividing cells (8,9). Moreover, it was demonstrated that during the proliferation of 3T3 cells stimulated with various growth factors, PFK activity immediately increased and lasted for 3 hrs in the absence of RNA or protein synthesis (9). Enzyme activation independent of protein synthesis appears to be involved in regulating fibroblast PFK activity (9). This is in general agreement with our observations that rising lymphocyte PFK activity was not inhibited by CHX. Lymphocytes, however, also have a CHX-sensitive phase which immediately precedes the period of increasing PFK activity. This apparent difference between our findings and the preceding one on 3T3 cells may be a reflection of the cell types used.

Although small lymphocytes and serum-starved 3T3 fibroblasts are both resting cells, the former may be more quiescent than the latter cells (10). It has been postulated that lymphocytes after Con A stimulation must undergo an activation period consisting of a host of steps to arrive at the stage where serum-deprived 3T3 fibroblasts are apparently arrested. Thereafter, cellular events leading to S phase may be similar in both types of cells. Early CHX treatment may be preventing Con A stimulated lymphocytes from progressing through these activation steps which may also include the synthesis of inactive PFK enzyme molecules.

This interpretation is consistent with the following observations: (1) the increase of PFK activity in 3T3 cells occurred immediately after mitogenic stimulation, whereas PFK activity did not increase until 8-10 hrs after Con A stimulation of lymphocytes; (2) once the increase in PFK activity began, CHX did not prevent the enzyme activity from rising in either 3T3 cells or lymphocytes.

The mechanism by which lymphocyte PFK activity increased after Con A stimulation in the absence of protein synthesis is unknown. Post-translational modification of preexisting enzyme molecules such as polymerization of inactive dimers into active tetramers (11-14) or changes in the phosphorylation state of the enzymes (15,16) are two possibilities. Further experiments to uncover the mechanisms may provide important information on the regulation of PFK activation as well as lymphocyte proliferation.

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